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## PHOEBUS AARON THEODOR LEVENE

1869-1940

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In the sudden death on September 6, 1940, of Phoebus Aaron Theodor Levene, American biochemistry lost a colleague who contributed beyond measure to the upbuilding of our science. He was one of those who in the early years of the century brought to America the best traditions of Europe to stimulate the sound development of science in this country. And in that development his own contributions played a brilliant part.

He was born in Russia, and graduated at the Imperial Military Medical Academy at St. Petersburg. Settling in New York in 1892, he practiced medicine until 1896, when his career was interrupted by tuberculosis. After recuperating at Saranac and Davos in Switzerland, he decided to devote his life to biochemistry. He therefore remained in Europe to study under the great masters of the day, Kossel, Drechsel, and Emil Fischer. Returning to America, he worked at Saranac in 1901-02 on the chemistry of the tubercle bacillus, and then entered the new laboratory of the State Pathological Institute. In 1905 Dr. Simon Flexner invited him to join the staff of the newly created Rockefeller Institute, and there he continued the extraordinary series of contributions to science which never slackened through all the rest of his life.

In 1920 Dr. Levene married Anna M. Erickson of Montana. Their home was a welcome center for a wide circle of friends, scientific, artistic, and literary, who found a congenial atmosphere in the hospitality of their versatile and sympathetic hosts.

Dr. Levene's publications, largely shared by his coworkers, have numbered over seven hundred. The fields that he explored included the following: the chemistry of the nucleic acids; of proteins and amino acids; of lipids; of carbohydrates and conjugated carbohydrates; of glycoproteins, which included especially a study of the chemistry of amino sugars; an investigation of the stereochemistry of natural products, which led to extensive studies on

stereochemical configurations and interrelationships of a wide range of simpler synthetic substances; the study of the mechanism and nature of the Walden rearrangement; studies on the isolation of the vitamin B complex; and, finally, during the last few years of his life, the chemistry of the gums and pectins.

To estimate his part in the advancement of science one must add to these fruitful studies his influence in developing young men. No one could work with him without being fired by his spirit, and a significant part of the growth of biochemistry in America is to be attributed to men who were trained and inspired in his laboratory. And he gave to his men not only scientific leadership, but warm personal interest; so that all who worked with him hold his memory in affection.

WALTER A. JACOBS

DONALD D. VAN SLYKE

# THE FERMENTATIVE DECOMPOSITION OF PURINES BY CLOSTRIDIUM ACIDI-URICI AND CLOSTRIDIUM CYLINDROSPORUM

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(Received for publication, June 24, 1941)

*Clostridium acidi-urici* is an anaerobic soil bacterium which grows readily in media containing salts of uric acid or certain other purines but which has little or no ability to decompose other types of organic compounds (Barker and Beck). The organism has also been shown to convert carbon dioxide into acetic acid (Barker, Ruben, and Beck, 1940). The present paper is concerned with the action of cell suspensions and growing cultures of *Clostridium acidi-urici* upon purines and other nitrogenous compounds. More specifically, we have determined the compounds attacked by this organism, the kinds and quantities of products formed, and the influence of various factors upon the kinetics of the reactions.

## Methods

The organisms used were two strains of *Clostridium acidi-urici* (Strains 5a, 9a) and one of *Clostridium cylindrosporum* (Strain HCl). The general characteristics of these strains are described elsewhere (Barker and Beck).

For growth experiments a medium of the following composition was used: distilled water, substrate 0.1 to 0.3 per cent, yeast autolysate 0.5 volume per cent,  $K_2HPO_4$  0.03 per cent,  $MgSO_4 \cdot 7H_2O$  0.005 per cent,  $CaSO_4$  0.25 volume per cent of a saturated solution,  $FeSO_4 \cdot 7H_2O$  0.0002 per cent, phenol red indicator 0.1 volume per cent,  $Na_2S \cdot 9H_2O$  0.01 per cent, and NaOH to pH 7.4 to 7.6. Both semisolid (0.1 per cent) agar and liquid media were used. Semisolid media were generally incubated in test-tubes without anaerobic seals, while liquid media were placed in completely filled glass-

stoppered bottles or in flasks filled to the neck and protected from oxygen by means of alkaline pyrogallol. All media were inoculated soon after being autoclaved and were incubated at 35°. Growth generally occurred, if at all, within 24 hours.

The organisms for cell suspension experiments were grown in a liquid medium similar to that described above with 0.25 per cent uric acid as the substrate and with 1.0 to 2.5 volumes per cent of yeast autolysate. 500 to 1000 cc. cultures protected from oxygen were heavily inoculated (1 to 10 volume per cent) and incubated for 12 to 16 hours at 35°. After this period of incubation growth is abundant and yet the bacteria are actively motile and usually still free of spores. Bacteria that have become immotile as a result of longer incubation are much less active. The cells were centrifuged and washed once with M/15 phosphate buffer solution, pH 7.1. Just before use this solution was boiled free of oxygen and 3 cc. of a 1 per cent  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  solution were added per 100 cc. The bacteria were resuspended in the same buffer, care being taken to minimize their exposure to oxygen. The final volume was 1.5 to 2.0 per cent of the original culture. Such suspensions usually contain 0.6 to 1.6 mg. of cell nitrogen per cc. (4.5 to 12.0 mg. of dry weight of cells per cc.). When not used immediately, they were stored in a refrigerator (5°) *in vacuo*. Stored suspensions retain almost their full urate-decomposing activity for at least 24 hours; after 3 days about one-third of the activity is lost.

*Compounds Decomposed*—Previous experiments (Barker and Beck) have shown that *Clostridium acidi-urici* grows vigorously in media containing uric acid but develops poorly or not at all in media containing complex nitrogenous materials such as tryptone or yeast extract in the absence of added purines. These observations indicate that the ability of the organism to attack nitrogenous compounds is very restricted. In order to determine just how great this specificity is and to obtain information bearing upon the mechanism of purine decomposition a survey was made of the ability of *Clostridium acidi-urici* to attack a large number of nitrogenous and a few non-nitrogenous compounds.

The tests were mostly carried out by incubating 1 cc. of a buffered (pH 7.6) cell suspension with 1 cc. of solutions of the test compounds for several hours *in vacuo* at 37°. Thunberg tubes were used as experimental vessels. Decomposition was judged by the formation of ammonia, which was estimated by titration follow-

ing distillation from a solution made alkaline with sodium borate. The behavior of the organisms towards the non-nitrogenous substrates was determined by testing for carbon dioxide production by means of Warburg's manometric apparatus.

All data on ammonia production are corrected for a blank without substrate. The activity of the cell suspensions was always checked by adding urate to one tube. With compounds that yielded no ammonia, the possibility that the negative result was due to toxicity was always tested by a separate experiment in which urate was mixed in equal amounts with the substrate in

TABLE I  
*Action of Cell Suspensions of Strain 9a on Purine Derivatives*

Substrate	Quantity	Period of incubation	Per cent conversion to ammonia
	<i>mg.</i>	<i>hrs.</i>	
Uric acid.....	4.00	2	91
Xanthine.....	1.28	11	98
Guanine.....	1.15	12	82
Hypoxanthine.....	2.00	14	96
Adenine.....	3.00	29	13
Adenosine.....	5.00	6	10
Guanosine.....	5.00	6	13
Adenylic acid.....	2.10	2	0*
Yeast nucleic acid.....	5.00	6	0*

\* Strain 5a converted 13 and 7 per cent of the nitrogen of adenylic acid and yeast nucleic acid, respectively, into ammonia in 18 hours.

question. None of the compounds studied appeared to be toxic in the concentrations used. Some compounds, because of their low solubilities, were used as saturated solutions containing an excess of the solid phase.

Table I illustrates the action of Strain 9a upon a number of purines and their derivatives. Similar results were obtained with Strain 5a or HCl or both, except as the contrary is indicated. In addition to the compounds listed in Table I, the following substances were tested and found not to be attacked (as judged by ammonia or carbon dioxide production) by any of the previously mentioned strains: purine,<sup>1</sup> urea, allantoin, uracil, caffeine, theo-

<sup>1</sup> The authors are greatly indebted to Dr. H. A. Krebs for providing a small sample of this compound, synthesized by E. Fischer.

bromine, parabanic acid, uramil, diethylbarbituric acid, isodialuric acid, urethane of 4,5-diaminouracil, theophylline, alloxan, alloxantine, hydantoin, creatine, imidazole, arginine, guanidine, glucose, pyruvic acid, glycolic acid, and three amino acid mixtures containing (1) leucine, lysine, aspartic acid, alanine, valine, and proline, (2) arginine, histidine, and cysteine, and (3) hydroxyproline, tyrosine, and glycine. These results apply when the substrates are supplied alone or are mixed with urate, with the exception that glycine is decomposed in the presence but not in the absence of urate. The behavior of glycine will be discussed below in some detail.

On the basis of the above results the compounds tested may be divided into three groups. The first group includes those compounds that are fairly rapidly and completely decomposed; namely, uric acid, xanthine, guanine, and hypoxanthine. The second group contains substances that are more slowly attacked by one or more strains and that yield only a small part of their nitrogen as ammonia; namely, adenine, adenosine, guanosine, adenylic acid, and yeast nucleic acid (Eastman Kodak Company). The third group includes all the remaining compounds which yield neither ammonia nor carbon dioxide and therefore presumably are not decomposed.

An inspection of these groups indicates the great specificity of *Clostridium aciduri*. The only compounds readily decomposed are free purines containing at least 1 oxygen atom. Adenine and purine were the only available purines lacking oxygen. The former is decomposed very slowly and incompletely, probably only the amino nitrogen atom being split off, while the latter is not attacked at all. Substitution of the hydrogens attached to nitrogen by methyl groups (caffeine, theophylline, theobromine) or by ribose (guanosine) greatly increases the stability of the resulting compound. The inability of the organism to act upon urea or allantoin, either in the presence or absence of urate, is of special interest, since these compounds are thereby eliminated from consideration as intermediates in the breakdown of purines. In this connection it may be recalled that in the aerobic decomposition of uric acid by bacteria (Liebert, 1909; Krebs and Eggleston, 1939) as well as by higher organisms (Fosse and Brunel, 1933; Brunel, 1937), allantoin has been shown to be an intermediate and urea is a normal end-product.

Previously described experiments (Barker and Beck) have shown that only those compounds that are rapidly decomposed by cell suspensions are suitable as substrates for growth.

*Glycine Decomposition*—It has already been mentioned that glycine is distinguished from all other compounds examined by being attacked only when urate is simultaneously decomposed. In order to clarify the interrelation between these two processes, a study was made of glycine decomposition as a function of the ratio of glycine to uric acid.

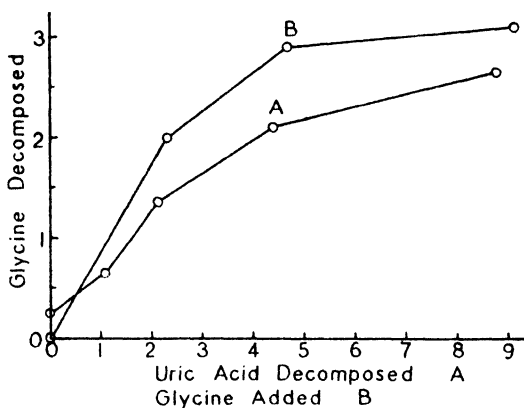


FIG. 1. The influence of urate and glycine supply upon the decomposition of glycine. Each unit represents 0.1 mg. of nitrogen. Curve A, total glycine nitrogen = 0.455 mg.; Curve B, total uric acid nitrogen = 0.875 mg. The total volume of solution in both experiments was 2.7 cc.

Curve A of Fig. 1 gives the results of an experiment in which the quantity of glycine was kept constant while the urate was varied. The period of incubation was sufficiently long (3.5 hours at 37°) to insure complete decomposition of the urate by the cell suspension. 0.455 mg. of glycine nitrogen was initially present in a total volume of 2.7 cc. The amount of glycine decomposed was calculated by subtracting the urate nitrogen  $\times$  0.96 from the total ammonia nitrogen formed. The factor 0.96 represents the recovery of urate nitrogen as ammonia in control experiments.

Curve A (Fig. 1) shows that in the absence of added urate only very little glycine is decomposed. The small ammonia production from glycine alone is actually greater in this experiment than in



other similar experiments, owing possibly to the presence of some urate in the cell suspension. The addition of a little urate causes an almost linear increase in glycine decomposition, while with larger additions the increase is relatively smaller. At a urate nitrogen-glycine nitrogen ratio of about 2:1 somewhat less than 60 per cent of the glycine is converted to ammonia. However, the curve of glycine decomposition as a function of available urate is still rising at this point, so that with an even greater ratio of urate to glycine the latter might well be completely decomposed. This possibility is supported by the data presented in Curve B.

Curve B of Fig. 1 gives the results of a similar experiment in which the glycine concentration was varied while the urate was kept constant at 0.875 mg. of N per 2.7 cc. Small amounts of glycine are almost completely decomposed, while with larger amounts the curve rapidly approaches an upper limit corresponding to about 1.5 moles of glycine per mole of urate.

The results of the two experiments may be summarized by saying that an increase in the available urate when the glycine is held constant increases both the percentage and the absolute quantity of glycine decomposed, while an increase in the concentration of glycine while the urate is kept constant increases the absolute quantity but decreases the percentage of glycine decomposed.

The dependence of glycine decomposition on the concentrations of both glycine and uric acid definitely indicates that a reaction occurs between glycine and urate or some product of urate breakdown. The exact nature of this reaction has not yet been established, though in view of the fact that glycine is activated by *Clostridium acidi-urici* as a hydrogen donor (see below), it is probable that glycine is oxidized, while urate or its products are reduced.

Glycine is similarly decomposed by Strain 9a in the presence of either xanthine or guanine, and by Strain HCl in the presence of urate.

*Products of Purine Decomposition*—The principal products (other than cell material) of the decompositions of uric acid, guanine, xanthine, and hypoxanthine by growing cultures and cell suspensions of *Clostridium acidi-urici* are ammonia, carbon dioxide, and acetic acid. The last was identified in the volatile acid fraction by the characteristic form of the crystals of sodium uranyl

acetate and was shown to be free of other volatile acids by Duclaux distillation. No neutral volatile products could be detected.

No attempt has been made to identify the products, other than ammonia, derived from the more slowly decomposed compounds of the second group (see above).

Quantitative data on the fermentation products of uric acid, guanine, xanthine, and hypoxanthine by cell suspensions are given in Table II. The experiments were carried out by incubating 50 mg. of substrate with 6 cc. of a buffered cell suspension at 37° *in vacuo*. At the end of the incubation period carbon dioxide was determined by the Van Slyke manometric method. Ammonia

TABLE II

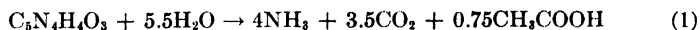
*Fermentation Products of Uric Acid, Guanine, Xanthine, and Hypoxanthine Formed by Cell Suspensions*

Substrate (50 mg.)	Strain No.	Period of incubation	Products per 100 moles substrate decomposed			Per cent recovery	
			CO <sub>2</sub>	CH <sub>3</sub> COOH	NH <sub>3</sub>	Carbon	Nitrogen
		<i>hrs.</i>	<i>moles</i>	<i>moles</i>	<i>moles</i>		
Uric acid.....	9a	12	340	72	397	96	99
“ “ .....	HCl	12	300	71	380	88	95
Guanine.....	9a	18	297	98	500	99	100
“ .....	5a	44	298	94	500	97	100
Xanthine.....	9a	29	264	101	411	93	103
Hypoxanthine.....	9a	36	199	127	370	91	92.5
“ .....	5a	17	(161)	118	380	(79)	95

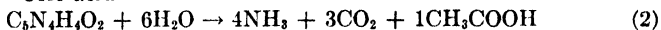
The values in parentheses are less reliable in view of possible errors in CO<sub>2</sub> determination.

and acetic acid were estimated by titration following steam distillation. All quantities are corrected for the products formed in a simultaneously incubated cell suspension without added substrate. The carbon and nitrogen recoveries are calculated on the assumption that the substrate was completely decomposed during the incubation period. Quantitative determinations showed this assumption to be correct for uric acid. For other purines suitable quantitative methods were not readily available. However, the high recoveries of nitrogen as ammonia indicate that decomposition was substantially completed except in the case of hypoxanthine which is much more slowly attacked than the other substrates.

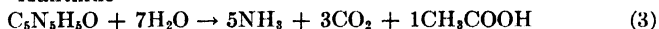
The data of Table II can be most easily evaluated by comparing the observed yields of products with the yields that would be obtained by a complete conversion of the various purines into ammonia, carbon dioxide, and acetic acid, according to the following equations.



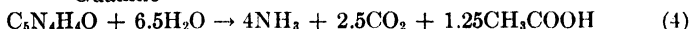
Uric acid



Xanthine



Guanine



Hypoxanthine

Such a comparison shows that the fermentations of uric acid, xanthine, and guanine by Strains 9a or 5a are rather closely described by Equations 1, 2, and 3, respectively. However, in the decompositions of uric acid by Strain HCl and of hypoxanthine by Strains 9a and 5a there are significant deviations from the equations; both the carbon and nitrogen recoveries are low. It will be shown later that in the uric acid decomposition by Strain HCl the discrepancy is due to the formation of an organic nitrogenous product which was not recognized at the time these experiments were carried out. The low recoveries of carbon and nitrogen from hypoxanthine are probably due to a different cause; namely, the incomplete utilization of the substrate. The possibility is, however, not altogether excluded that other unidentified products are formed in this case also. But in spite of some evident discrepancies it may be said that all four purines are fermented by cell suspensions at least approximately in accordance with the above equations.

When the growing culture technique was used, very similar results were obtained (Table III). Here again the most significant deviations from the equations occurred in the fermentation of hypoxanthine by Strain 9a and of uric acid by Strain HCl. The probable explanations are the same.

*Glycine Formation from Uric Acid by Strain HCl*—It has been shown in Tables II and III that uric acid is almost completely converted into ammonia, carbon dioxide, and acetic acid by Strains 9a and 5a, while with Strain HCl the recoveries of nitrogen and car-

bon in the form of these same products are always low. Since the missing nitrogen and carbon might well be present in one or more compounds whose identification would throw light upon the mechanism of the breakdown of uric acid, the study of such compounds appeared to be of special interest. Because of the great ease with which nitrogen may be estimated, attention was given primarily to the isolation of a compound containing this element.

To obtain a considerable quantity of the unidentified nitrogenous compound, 5 liters of the usual growth medium, containing 0.3 per cent uric acid and 0.2 volume per cent of yeast autolysate

TABLE III

*Fermentation Products of Uric Acid, Guanine, Xanthine, and Hypoxanthine Formed by Growing Cultures*

All cultures were incubated at 35° until visible development ceased.

Substrate	Strain No.	Products per 100 moles substrate decomposed			Per cent recovery	
		CO <sub>2</sub>	CH <sub>3</sub> COOH	NH <sub>3</sub>	Carbon	Nitrogen
		<i>moles</i>	<i>moles</i>	<i>moles</i>		
Uric acid .....	9a*	348	67	398	97	99.5
“ “ .....	5a	342	60	380	92	95
“ “ .....	Sl	356	63	384	96.5	96
“ “ .....	HCl*	331	53	351	87	88
Guanine .....	9a*	275	112	490	99	98
“ .....	5a	305	104	500	104	100
Xanthine .....	9a*	273	106	374	97	95
Hypoxanthine .....	9a*	199	105	329	82	82

\* Averages of two or more experiments.

were inoculated with Strain HCl and incubated for 6 days. It should be noted that the minimum concentration of yeast autolysate which would allow good growth was used so that the nitrogenous constituents of the yeast would interfere as little as possible with the isolation of the nitrogenous product of uric acid decomposition. Probably because of the low concentration of yeast autolysate, the uric acid was not completely fermented in this experiment, about one-fourth of the initial amount remaining at the end of the incubation period.

As in previous experiments with Strain HCl ammonia accounted

for only part (94.4 per cent) of the fermented uric acid. Although the remaining nitrogen was not as much as had been expected on the basis of earlier results with growing cultures (Table III), there still remained some hope of identifying this fraction, since it contained almost 3 times as much nitrogen as the added yeast autolysate.

In order to facilitate the isolation and identification of the unknown nitrogen compound, ammonia and residual urate were removed from the fermented medium, the former by vacuum distillation at 55–60° in the presence of a slight excess of  $\text{Ba}(\text{OH})_2$  (barium phosphates and carbonates were first filtered off), the latter by subsequent precipitation with  $\text{Ag}_2\text{SO}_4$  at pH 4. The urate- and ammonia-free solution contained a total of 288 mg. of nitrogen.

The nature of the nitrogen compounds in this solution was investigated by making a number of qualitative tests. It at once became apparent that the solution contained urea, which was identified by the formation of xanthidrol urea and by the liberation of ammonia in the presence of urease. A quantitative estimation of urea by the urease method showed that 41 mg. of urea nitrogen were present.

There were two possibilities as to the origin of this urea. It might be a product of fermentation or it might have been formed during removal of ammonia by the action of alkali upon the residual uric acid. The latter alternative seemed the more probable, since uric acid is known to liberate urea slowly under the conditions used. In a second, similar experiment only exceedingly little urea was obtained when care was taken to remove ammonia under conditions precluding the decomposition of uric acid (distillation *in vacuo* at 20°). It seems safe to conclude that urea is not a product of biological action.

Besides urea, the urate- and ammonia-free solution also contained 219 mg. of amino acid nitrogen. The latter was estimated by the Van Slyke nitrous acid method with a reaction time of 4 minutes at 25°. By using longer reaction times the nitrogen liberated was progressively increased, until after 2 hours it became approximately equal to the total nitrogen of the solution. This result indicated the presence of compounds with amino groups in other than the  $\alpha$  position. Such slowly reacting amino groups are present in

urea as well as in certain nitrogen compounds of the yeast autolysate. Since the quantity of slowly reacting amino nitrogen not attributable to urea was small (33 mg.) and since it was certainly derived largely if not entirely from the yeast autolysate, this fraction was not further investigated.

The nitrogen compound of greatest interest was clearly that possessing the rapidly reacting  $\alpha$ -amino group, which made up roughly 72 per cent of the total nitrogen. Various lines of evidence which need not be detailed here indicated that this compound might be glycine. Attempts to demonstrate and estimate glycine directly by means of the specific potassium trioxalatochromiate reagent (Bergmann and Fox, 1935) were unsuccessful, possibly because of the small amount of the nitrogen compound at our disposal. But eventually we succeeded in separating a crystalline amino compound having the general properties of glycine by making use of the solubility of its hydrochloride in 95 per cent ethanol. After the ethanol was distilled off and the crystalline residue dissolved in water, the remaining inorganic ions were largely removed with  $\text{Ag}_2\text{SO}_4$  and  $\text{Ba}(\text{OH})_2$ . The solution was then concentrated to a small volume and 10 volumes of absolute ethanol were added with the result that a crystalline precipitate formed. This was washed with absolute alcohol and dried *in vacuo*. The nitrogen content of the crystalline material was 41 per cent of the total nitrogen of the original solution.

The crystals were analyzed for total nitrogen by the micro-Kjeldahl method, for amino nitrogen by the Van Slyke nitrous acid method, for total carbon by the wet combustion method of Van Slyke, Page, and Kirk (1933), and for carboxyl carbon by the ninhydrin method of Van Slyke and Dillon (1937). The results are given in Table IV which includes comparable data for glycine.

The analytical data clearly indicate that the crystalline material is mainly glycine, probably contaminated with small amounts of other amino acids. These impurities could doubtless have been removed by further recrystallizations but this was not attempted because of the small amount of material available.

The identification of glycine was further confirmed by observations on the optical properties of the crystals, by the characteristic appearance of the copper salt (Cunningham, MacIntyre, and Kirk, 1936), by benzylation to give hippuric acid with a melting point of

186° (uncorrected), and by a specific biological method involving the use of an anaerobic bacterium<sup>2</sup> capable of fermenting glycine.

Glycine having been identified as a product of uric acid decomposition, the next problem was to estimate the quantity of this compound. To this end the trioxalatochromiate method was tried but in our hands it did not give satisfactory results. So for want of a more specific method the quantity of glycine was calculated from amino acid nitrogen determinations on the assumption that it was the only amino acid formed. In the experiment under consideration the 219 mg. of amino nitrogen (4 minutes reaction time at 25°) present in the fermented medium were corrected for the initial amino nitrogen of the yeast autolysate (26 mg.), for

TABLE IV

*Analyses on Nitrogenous Product of Uric Acid Fermentation*

The results are expressed as per cent of dry weight, on an ash-free basis.

Analysis	Unknown	Glycine
Total N . . . . .	18.1	18.7
NH <sub>2</sub> -N . . . . .	18.7	18.7
COOH-C . . . . .	15.1	16.0
Total C . . . . .	31.8	32.0

urea nitrogen (4 mg.), and for the high results (105 per cent) always obtained for glycine by the usual nitrous acid method. The final calculated value was 180 mg. of glycine nitrogen or 964 mg. of glycine. This is equivalent to 4.9 per cent of the nitrogen of the uric acid fermented. Since in this experiment ammonia accounted for 94.4 per cent of the substrate nitrogen, the total nitrogen recovery was 99.3 per cent. This figure does not include the nitrogen in the bacterial cells, which has been shown to represent 0.5 to 1 per cent of the substrate nitrogen.

In a second large scale growth experiment with Strain HCl, during which 23 gm. of uric acid were fermented, 94.2 per cent of the nitrogen was recovered as ammonia and 3.1 per cent as glycine when the method of glycine estimation described above was used.

Glycine is also formed by the action of cell suspensions of Strain

<sup>2</sup> A study of this organism will be published later. We are indebted to Mr. B. C. Cardon for carrying out the biological test.

HCl on urate and xanthine. In one experiment with urate the yield of glycine nitrogen (4.7 per cent) was almost the same as was previously obtained with growing cultures. With xanthine the yield of amino nitrogen (1.5 to 2 per cent) was smaller, but still significant. In contrast to these results with Strain HCl, cell suspensions of Strain 9a produced no detectable amino nitrogen from urate. This was of course to be expected on the basis of the high recoveries of ammonia already reported.

*Experiments on Rates of Purine Fermentations. Application of Manometric Technique*—In studying the rates of purine fermentations, we found the Warburg manometric technique generally satisfactory. For most experiments 14 to 15 cc. single arm vessels were used and in each was placed 0.2 to 0.5 cc. (the smaller volume is usually better) of a cell suspension prepared as previously described, 0.2 cc. of 0.5 M phosphate or bicarbonate buffer (depending upon the type of experiment), 0.2 to 0.8 cc. of M/70 to 0.1 M substrate solution, and boiled water to give a total volume of 2.0 cc. The gas phase consisted of oxygen-free  $N_2$  or  $H_2$ . Considerable care must be taken to remove oxygen completely, for the cells are soon inactivated by this gas; however, this effect is evidently reversible over short periods of time, since brief contact with oxygen while the manometers are being set up appears to have no deleterious effect on the later activity of cell suspensions. Uric acid was used in the form of the lithium salt because of its greater solubility. Other purines were dissolved in weak NaOH and were neutralized just before use to give supersaturated solutions or finely divided precipitates. All experiments were carried out at 37.1°.

To observe the rate of urate fermentation by the manometric technique two methods were employed. One requires the use of a bicarbonate buffer and a gas phase containing 5 per cent or more carbon dioxide and involves measurements of carbon dioxide uptake resulting from the excess alkali produced in urate decomposition. This is the reverse of the usual method of measuring acid production. The second method, which is simpler, more sensitive, and more generally applicable, involves the measurement of carbon dioxide production in vessels containing a phosphate buffer and an  $N_2$  atmosphere.

The first method is mentioned chiefly because of a curious phenomenon which can be easily observed only with this technique.



This phenomenon is illustrated in Fig. 2. It can be seen that the pressure change due to the cell suspension alone is negligible but that as soon as urate is added carbon dioxide begins to be absorbed at a rate which increases for a time and then declines rapidly as the substrate is exhausted. The point of special interest is that the pressure change does not immediately drop to zero but first changes sign, indicating the evolution of gas. The more urate is fermented the greater is the magnitude and duration of this effect.

Now the evolution of gas in the experiment under consideration must be due to an increase in the acidity of the medium and a

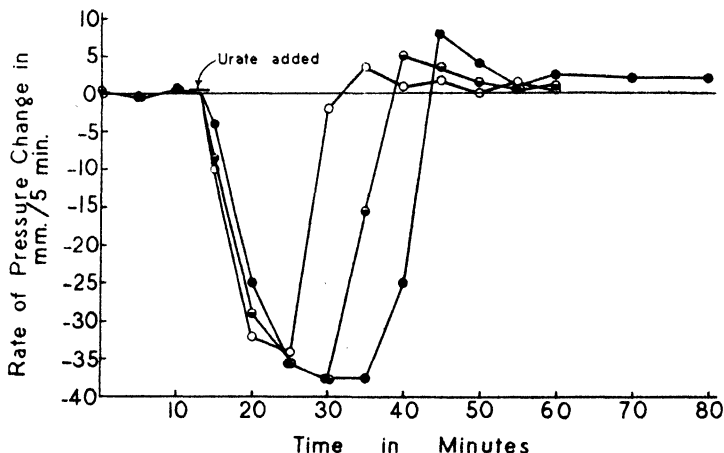


FIG. 2. Manometric changes accompanying the decomposition of uric acid by Strain 9a in a bicarbonate-carbon dioxide buffer system. Quantities of 0.02 M urate per vessel are as follows: ○ 0.3 cc., ◐ 0.5 cc., ● 0.7 cc. Each vessel contains 0.4 cc. of cell suspension.

consequent formation of carbon dioxide from bicarbonate. The production of carbon dioxide alone, unaccompanied by an increased acidity, could not cause the observed positive pressure change, since the system is buffered with respect to this gas. The only other possible explanation for the effect is the formation of an insoluble gas like  $H_2$  or  $N_2$ . That this does not occur was proved by special experiments in which the bicarbonate was replaced by a phosphate buffer and NaOH was placed in the central well. Under these conditions no net pressure change was observed.

To explain the increase in acidity it seemed important to learn

whether this change occurs before or after the ammonia is all liberated. This point was studied by determining ammonia at various times during a manometric experiment in which the pressure changes were simultaneously followed. The results show that ammonia production stopped almost exactly at the moment when the pressure readings changed sign.

A further significant fact is that the magnitude of the positive pressure change is highly dependent upon the quantity of bacteria used; the fewer the bacteria, the smaller is the effect. Indeed, with 0.1 cc. of cell suspension per manometer vessel, the effect is scarcely observable; only with 0.3 to 0.5 cc. of cell suspension is it conspicuous.

All of the above observations can be most readily explained by assuming that the bacteria are much more permeable to ammonia than to acetic acid under the existing conditions. According to this view the ammonia formed from the decomposition of uric acid in the interior of the cells escapes to the surrounding medium more rapidly than does the acetic acid, leaving the cell contents relatively more acid and the medium more alkaline. The subsequent release of acetic acid results in a decline in the alkalinity of the medium, which in turn liberates carbon dioxide and causes the sign of the pressure reading to change from negative to positive.

Fig. 2 also shows that the rate of urate fermentation is largely independent of urate concentration over a considerable range. The slight apparent differences in rate in the descending parts of the curves are due to the fact that the substrate was not mixed with the cells in all vessels at exactly the same time.

*Influence of pH*—Experiments dealing with the influence of pH on the rate of urate decomposition by cell suspensions were carried out by using 0.2 cc. of 0.5 M phosphate buffer and 0.6 cc. of 0.02 M lithium urate per vessel. Since the fraction of the total carbon dioxide entering the gas phase from a buffered solution is a function of pH, the rate of pressure change cannot be taken as a direct measure of the rate of decomposition. The results given in Fig. 3 are consequently calculated from the reciprocals of the times required for complete decomposition of a given amount of urate. The rate is expressed in c.mm. of  $\text{NH}_3$  per hour per mg. of dry weight of bacteria ( $\equiv Q_{\text{NH}_3}$ ). 1 mole of  $\text{NH}_3$  is equivalent to  $22.4 \times 10^6$  c.mm.

It can be seen (Fig. 3) that the maximal rate of fermentation occurs at about pH 7.3 and falls off rapidly on either side of this maximum. Below pH 6.4, where the buffer capacity of the phosphate is relatively low, the pH will be increased appreciably by the alkaline products of urate decomposition. Consequently the average pH during decomposition will be higher than the initial pH which has been used to plot the experimental data. Unfortunately, the final reaction was not determined. The solid curve in Fig. 3, which was drawn to allow for this shift of pH, probably gives a

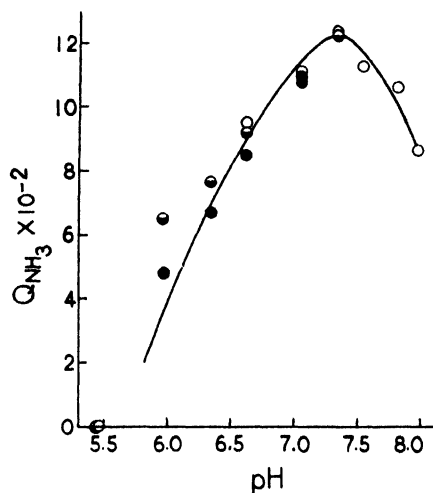


FIG. 3. Influence of pH on the rate (c.mm. of  $NH_3$  per hour per mg. of dry weight of bacteria) of urate decomposition. The variously shaded circles represent data of independent experiments.

truer picture of the rate-pH relation than do the experimental points.

The absolute rate of urate decomposition at the optimal pH is very high, being comparable to the rates of serine and aspartic acid deamination catalyzed by *Bacterium coli* (Gale and Stephenson, 1938).

*Influence of Carbon Dioxide*—Previous experiments (Barker, Ruben, and Beck, 1940) have shown that when *Clostridium acidurici* is allowed to act upon uric acid in the presence of radioactive carbon dioxide the acetic acid formed contains radioactive carbon

in both the carboxyl and methyl groups. A possible interpretation of this result is that the decomposition of uric acid by this organism is an oxidation in which carbon dioxide acts as the ultimate hydrogen acceptor and is reduced to acetic acid. Now if this interpretation is correct, one might expect the rate of decomposition to be in some degree dependent upon carbon dioxide concentration in the sense that lowering the latter would decrease the rate. Experiments were therefore carried out to see whether such an effect could be observed.

Since preliminary observations indicated that a retardation of urate decomposition can be observed, if at all, only at very low carbon dioxide tensions, the final experiments were arranged to produce this condition. As reaction vessels the tubes described by Johnson (1937) for use in a modified Thunberg technique were employed. These tubes have two arms in which separate components of a reaction mixture can be placed and aerated with a stream of gas prior to mixing. In one arm were placed 3 cc. of a  $\text{CO}_2$ -free solution of 0.025 M lithium urate, in the other a cell suspension in 4.3 cc. of  $\text{CO}_2$ -free 0.093 M phosphate buffer, pH 6.4. A buffer near the acid limit of urate decomposition was chosen in order to facilitate the removal of carbon dioxide. The gas used for this purpose was  $\text{CO}_2$ -free and  $\text{O}_2$ -free  $\text{H}_2$ ; the rate of bubbling was about 3 liters per minute. After gas was passed through the tubes for 45 minutes, the urate was mixed with the cell suspension. Three tubes were aerated with  $\text{H}_2$  during the entire incubation period (about  $\frac{1}{2}$  hour at  $37^\circ$ ), while the aeration of three other tubes was stopped immediately after mixing. The latter were shaken at frequent intervals to prevent sedimentation of the cells. The progress of the decomposition was estimated by parallel experiments with Warburg's manometric apparatus and the incubation period was chosen so that about half of the urate would be decomposed. The reaction was then stopped instantly and almost simultaneously in all tubes by addition of sulfuric acid to pH 1. The quantity of uric acid decomposed was calculated from the ammonia formed.

The result was completely negative; the rate of urate breakdown in the tubes from which carbon dioxide was continuously removed by vigorous aeration with hydrogen was as high or a little higher than in the unaerated control tubes. It must be emphasized,

however, that this negative result does not disprove the hypothesis that carbon dioxide acts as an oxidizing agent. The only conclusion to be drawn is that the removal of carbon dioxide in these experiments was not sufficiently complete to retard urate decomposition. That this should be so is quite understandable in view of the fact that the breakdown of urate releases carbon dioxide within the bacterial cells, from where it can escape only by the slow process of diffusion.

*Relative Rates of Decomposition of Various Purines*—A few observations were made on the relative rates of decomposition of uric acid, guanine, xanthine, and hypoxanthine by cell suspensions of Strain 9a. The cells were grown on a urate medium and the experiments were carried out by the manometric method. The media were all buffered with phosphate to pH 7.1. Relative rates were calculated from the reciprocals of the times required for complete decomposition of a definite quantity of substrate as indicated by cessation of gas production.

The results show that the relative rates of decomposition are approximately urate 100, guanine 90, xanthine 55, and hypoxanthine 1. Hypoxanthine decomposition evidently involves an adaptive enzyme, since during the first 90 minutes no carbon dioxide was produced, while during a further 5 hour incubation period the rate of gas production gradually increased to 1 to 2 per cent of that for urate. A still longer incubation must result in a further increase in rate, since experiments already reported (Tables II and III) show that the same organism can ferment considerable quantities of hypoxanthine in 14 to 36 hours at 35°. The enzymes responsible for the decompositions of xanthine and guanine appear to be constitutive, since the maximum rates of gas evolution were reached almost immediately after addition of these substrates. It should be mentioned that the previously reported (Barker, Ruben, and Beck, 1940) induction period in the decomposition of guanine and urate is not generally of importance, since it occurs only under very special conditions. The rates of breakdown of these compounds by active cell suspensions are not influenced by the presence of glycine.

A single experiment was carried out to see whether the rates of decomposition are additive when two substrates are simultaneously supplied. This is the case for mixtures of uric acid and

xanthine; the time required for the decomposition of each compound is independent of the presence of the other. It is also of interest that hypoxanthine, which is itself very slowly attacked, does not inhibit the breakdown of uric acid when both are present in the same concentration (0.003 M). All these results suggest that a different enzyme catalyzes the breakdown of each purine.

*Dehydrogenase Activity*—Methylene blue reduction in the presence of cell suspensions and suitable substrates may under certain circumstances be taken as an index of dehydrogenase activity. The following experiments were undertaken to find out what

TABLE V  
*Relative Rates of Methylene Blue Reduction by Various Compounds in Presence of Strain 9a*

Substrate	Rate	Substrate	Rate
Glycine.....	100	Hypoxanthine.....	3.5
Amino acid Mixture C*.....	100	Uric acid.....	3.1
Xanthine.....	33	“ “ + hypoxanthine..	3.5
Guanine.....	21	Adenylic acid.....	2.9
Yeast autolysate (10%).....	13	Adenine.....	2.7
Adenosine.....	8	Yeast nucleic acid.....	2.5
Guanosine.....	7.5	Ethyl alcohol.....	2.2
Amino acid Mixture B*.....	4.1	Hydrogen.....	1.0
“ “ “ A*.....	3.1	Blank.....	0.8

\* Mixture A, *dl*-leucine, *d*-lysine hydrochloride, *l*-aspartic acid, *dl*-alanine, *dl*-valine, *l*-proline; Mixture B, *d*-arginine hydrochloride, *l*-histidine hydrochloride, cysteine hydrochloride; Mixture C, *l*-hydroxyproline, *l*-tyrosine, glycine.

compounds are activated as hydrogen donors by *Clostridium acidurici* and to determine their relative rates of dehydrogenation.

The experiments were conducted by mixing 1 cc. of an approximately 0.002 M solution of the compound to be tested, 0.5 cc. of cell suspension, and 0.5 cc. of 0.1 M phosphate buffer solution, pH 7.6, in a modified Thunberg tube. 0.3 cc. of 0.002 M methylene blue was placed in the side arm and the tube was thoroughly evacuated. The relative rates of reduction (glycine = 100) at 37° were calculated from the reciprocals of the times required for decolorization. Table V summarizes the data of several experiments.

The most striking result is the very rapid reduction of methylene blue by glycine and by the amino acid Mixture C which contains glycine as the active constituent. This action of glycine is evidently quite specific, since other amino acids are dehydrogenated much more slowly if at all. The possible significance of the activation of glycine will be discussed later.

Among the purines, only xanthine and guanine cause the reduction of methylene blue at a fairly rapid rate. Uric acid acts much more slowly. It may at first seem strange that uric acid, which from other experiments is known to be the substrate par excellence, is so inactive in this test. The explanation undoubtedly is that the breakdown of uric acid (and other fermentable substrates) results in the formation of intermediate hydrogen acceptors as well as hydrogen donors. The former then compete with methylene blue for the available hydrogen and consequently prolong the decolorization time. Under such conditions the rate of methylene blue reduction obviously bears no simple relation to "dehydrogenase activity." There is, however, one significant conclusion that can be drawn from the reduction rates of xanthine, hypoxanthine, and uric acid; namely, that xanthine oxidase is not present in these organisms or, if present, is not active in the processes of decomposition taking place. Green (1934) has shown that in the absence of oxygen the enzyme xanthine oxidase converts equimolar amounts of uric acid and hypoxanthine into xanthine. Since with *Clostridium acidi-urici* xanthine reduces methylene blue much more rapidly than either urate or hypoxanthine, one would expect, if xanthine oxidase is present, that urate plus hypoxanthine should act more rapidly than either compound alone. Actually the mixture behaves just like hypoxanthine rather than xanthine. Therefore xanthine oxidase is not involved.

#### DISCUSSION

The decomposition of uric acid and other purines by *Clostridium acidi-urici* and similar anaerobic bacteria is of special biochemical interest because of the fact that the mechanism of the process is evidently very different from that involved in the breakdown of these compounds by certain aerobic microorganisms, higher plants, and animals. So far as is now known all these latter organisms carry out oxidative decompositions involving the formation of allantoin through the action of the enzymes xanthine

oxidase and uricase. The allantoin is then further broken down to give urea either by hydrolytic or oxidative reactions, depending upon the organism concerned. With the anaerobic bacteria, on the contrary, oxygen is not utilized, urea is not formed, and the enzymes mentioned above almost certainly play no part in purine breakdown.

In examining the available information on the mechanism of anaerobic purine decomposition two distinct though closely related problems require consideration. One concerns the chemical nature of the process as a whole. Does it involve oxidation-reduction reactions between purine or other organic molecules or is it essentially an oxidation of purines by means of carbon dioxide? The other problem concerns the transformations undergone by the individual purine molecules. What intermediate compounds and what enzymes are involved?

First let us consider the evidence bearing upon the general chemical nature of these metabolic processes. The hypothesis that they represent complete oxidations of purines by means of carbon dioxide which is reduced to acetic acid was first suggested by the formation of more than 1 mole of acetic acid per mole of hypoxanthine decomposed and is supported by previously reported experiments with radioactive  $C_{11}$  (Barker, Ruben, and Beck, 1940). It was observed that when either uric acid, guanine, or hypoxanthine is decomposed in the presence of radioactive carbon dioxide the resulting acetic acid contains radioactive carbon in both the methyl and carboxyl groups. This indicates that acetic acid is formed by reduction of carbon dioxide. The evidence, however, is not conclusive because of the possibility that simple exchange reactions (involving not acetic acid itself but rather some intermediate compound) could bring about the same result. An independent method of demonstrating the postulated rôle of carbon dioxide would be to show that the concentration of this substance controls the rate of purine decomposition. Unfortunately, all attempts to observe such an effect have given negative results. Though this in itself certainly does not disprove the hypothesis that carbon dioxide acts as a specific oxidant, it must be admitted that the evidence in favor of this view is not conclusive. The formation of 1.25 moles of acetic acid per mole of hypoxanthine could be explained in other ways.

The available information concerning the anaerobic transforma-



tions of uric acid or other purines is still fragmentary. Most conclusive is the evidence against involvement of the previously recognized enzymes and intermediates of purine breakdown, such as xanthine oxidase, uricase, allantoinase and allantoin, urease and urea. The absence of xanthine oxidase from *Clostridium acidi-urici* is made probable by the failure of the organism to attack purine itself and by the methylene blue reduction experiments which indicate that hypoxanthine and uric acid do not react to give xanthine. Uricase is eliminated from consideration by the anaerobic nature of the process and the probable non-occurrence of allantoin as an intermediate. Allantoinase and allantoin as well as urease and urea are excluded by the inability of the organisms to attack allantoin and urea, respectively. Finally the failure of urea to accumulate in the absence of a urea-decomposing enzyme eliminates allantoicase as well as all other enzyme systems leading to the formation of urea. All the negative evidence leads irresistibly to the conclusion that the mechanism of anaerobic bacterial purine breakdown differs fundamentally from the better known aerobic process.

Positive evidence on the mechanism of anaerobic purine decomposition is concerned entirely with the behavior of glycine. There can be no doubt that glycine is an intermediate. It accumulates in considerable quantities during the decompositions of uric acid and xanthine by Strain HCl and is decomposed by both Strains HCl and 9a when a purine is simultaneously available. The failure of glycine to accumulate in cultures of Strain 9a is very probably due to its more rapid decomposition by this organism and cannot be taken as evidence against its intermediate formation.

Although the occurrence of glycine as an intermediate is well established, it must be emphasized that there is no certainty that it is formed in purine breakdown rather than in carbon dioxide reduction. Indeed the latter alternative is strongly suggested by previously reported experiments with radioactive carbon which showed that a considerable part, as much as 40 per cent, of the added carbon dioxide carbon is converted into a water-soluble, non-volatile material that could be glycine. Further experiments to test this point are in progress.

Evidence regarding the breakdown of glycine is more definite than that regarding its origin. Experiments by the Thunberg

technique prove that glycine behaves as a very active reducing agent under the influence of the bacteria and it is therefore probable that also in a normal purine fermentation it is oxidized to yet undetermined products. The activation of glycine as a hydrogen donor is of some interest in itself, since other anaerobes like *Clostridium sporogenes* and *Clostridium botulinum* appear to use glycine only as a hydrogen acceptor.

In conclusion, mention should be made of the possibility of using suspensions of these bacteria for the qualitative and quantitative determination of free purines. Although only preliminary trials have so far been made in this direction, the specificity of the organisms is such as to give promise of the development of a useful method. The only compounds known to be rapidly and completely decomposed are uric acid, guanine, and xanthine. The complete decomposition of 1 mg. of uric acid, for example, requires only about 10 to 15 minutes under conditions suitable for manometric experiments. Any of the purines mentioned above could be estimated quantitatively, if present separately, by determining either the ammonia or the carbon dioxide formed by decomposition, and the individual compounds could be identified by finding the relative amounts of ammonia, carbon dioxide, and acetic acid. The simultaneous determination of the three purines in mixtures could also be carried out with a knowledge of the absolute and relative quantities of the products. Probably the only important interfering substance would be glycine, from which the purines can be readily separated by silver precipitation. Hypoxanthine, adenine, and other purine derivatives are decomposed so much more slowly than uric acid, guanine, and xanthine by cells grown on urate that they would not interfere.

#### SUMMARY

1. The uric acid-fermenting anaerobic bacteria, *Clostridium acidi-urici* and *Clostridium cylindrosporum*, are able to decompose only a very few organic compounds, all of which, with the exception of glycine, are purine derivatives. Uric acid, xanthine, and guanine are broken down rapidly and completely by cell suspensions and growing cultures, while hypoxanthine and some other compounds liberate their nitrogen slowly and generally incompletely.

2. The products of anaerobic purine decomposition by *Clostridium aciduri* are ammonia, carbon dioxide, and acetic acid, while *Clostridium cylindrospor* forms in addition small amounts of glycine. Both organisms decompose glycine when a fermentable purine is simultaneously available but not otherwise. Glycine is also activated as a strong hydrogen donor, and there are good reasons for believing that it represents an intermediate in either purine breakdown or carbon dioxide reduction.

3. The enzyme systems and intermediate compounds known to be involved in purine breakdown by animal tissues, plants, and aerobic microorganisms are evidently not involved in the disassimilation processes of these anaerobic bacteria. Although the detailed mechanism of these processes is largely obscure, there are reasons for believing that they represent oxidations in which carbon dioxide acts as the ultimate hydrogen acceptor and is reduced to acetic acid.

4. Data are presented on the influence of various factors on the rate of purine decomposition.

5. The possibility of using these bacteria for the quantitative and qualitative determinations of uric acid, guanine, and xanthine is pointed out.

The authors wish to express their appreciation to Professor F. W. Allen, Professor T. E. Rawlins, and Professor C. L. A. Schmidt for helpful suggestions, particularly with regard to the isolation and identification of glycine, and to Dr. J. J. Eiler and Dr. H. A. Krebs for contributing various purine and pyrimidine derivatives.

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## PREPARATION AND PROPERTIES OF CUCUMBER VIRUS 4

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A substantial relationship between cucumber viruses 3 and 4 and certain members of the tobacco mosaic virus group may be demonstrated by a comparison of their physicochemical properties and by serological tests (1-3). However, the test which would establish conclusively kinship between these nucleoproteins, namely the immunization of a plant diseased with cucumber virus 3 or 4 against infection from a strain of tobacco mosaic virus or the reverse, has been entirely lacking, for no host common to the two groups of viruses has thus far been found (1, 2, 4).

A comparative study of cucumber viruses 3 and 4 and strains of tobacco mosaic virus has been undertaken in an effort to find a chemical explanation for the similarities and differences existing between these related nucleoproteins. Moreover, a study of the cucumber viruses is of unique interest in connection with the relationship between host and infectious agent, for, contrary to the usual case with plant viruses, cucumber viruses 3 and 4 have been found transmissible in but one family of plants (4).

Earlier experiments with purified preparations of cucumber viruses 3 and 4 have been comparatively limited, and the data presented have dealt chiefly with cucumber virus 3 (2, 5, 6). Further, in most of these investigations chemical methods for purification were employed. It is known now that such methods may yield preparations which contain variable amounts of altered virus and show proportionally less activity than preparations obtained by physical means alone (7-10). A description of the isolation and purification of cucumber virus 4 by physical methods and of the properties of the purified virus is given in the present paper.

*Preparation of Virus*—Young cucumber plants were inoculated with cucumber virus 4 by rubbing one leaf on each plant with a gauze pad saturated with infective juice. This juice was obtained from a cucumber plant showing the typical yellow mottling produced by cucumber virus 4 in members of the Cucurbitaceae. About 1 month after inoculation the plants were harvested and placed in a room kept at  $-12^{\circ}$ . After a few days the frozen plants were ground, 3 per cent by weight of dipotassium phosphate in a 50 per cent solution was thoroughly mixed with the pulp, and after about 2 hours the juice was expressed from the cold but completely thawed pulp. This juice was passed through a celite filter to remove coarse particles of green pigment and extraneous matter and then the virus was sedimented in the form of pellets by centrifugation at 20,000 to 30,000 R.P.M. for 30 minutes. The supernatant liquid, which was practically virus-free, was discarded and the pellets were dissolved in small amounts of distilled water. The combined solutions of virus pellets were spun at about 3000 R.P.M. on an angle centrifuge for 30 minutes to remove green pigment and insoluble colloidal matter. The supernatant liquid, which contained the virus, was then returned to the high speed centrifuge and the process was repeated about three times, a longer period being allowed for the high speed centrifugation as the virus became more concentrated. In many cases it was found possible to effect a better separation of green pigment from the virus by dissolving the pellets obtained by the first two high speed centrifugations in 0.1 M phosphate buffer at pH 7 and then using distilled water as a solvent for the pellets of the last two centrifugations. All of the preparations used for elementary analyses were further purified by dialysis against flowing distilled water for 48 hours.

Yields of purified virus ranged from 0.1 to 0.4 gm. per liter of expressed juice as compared with 2 to 2.5 gm. per liter ordinarily obtained for tobacco mosaic virus from the juice of diseased Turkish tobacco plants. The yield of virus appeared to depend somewhat upon growing conditions, and especially upon the interval of time between inoculation and harvesting of the plants. The lower yields of virus were obtained from plants harvested 3 or 4 weeks after inoculation and the higher amounts were secured from plants collected 4 to 6 weeks after inoculation.

*Activity of Virus Preparations*—The infectivity of typical preparations of cucumber virus 4 was tested in each case by rubbing the leaves of young cucumber plants with 1 ml. of solutions containing various amounts of nucleoprotein, and noting the greatest dilution at which systemic infections were obtained. The results of three tests of this type reported for chemically purified cucumber virus 4 showed that the smallest amount of virus required to produce infection varied from  $10^{-8}$  to  $10^{-10}$  gm. (2). Table I shows the results of similar tests made with cucumber virus 4 prepared by the physical methods of filtration and differential centrifugation described above. Infections were noted for as little as  $10^{-12}$  gm. of virus. Although it is hazardous to compare the results of

TABLE I

*Infectivity of Purified Preparations of Cucumber Virus 4*

Two plants were used to test each dilution of virus, and 1 ml. of virus solution was used to inoculate each plant. + indicates systemic infection; — signifies that the plant remained healthy.

Preparation No.	Dilution, gm. virus per ml.					
	$10^{-7}$	$10^{-8}$	$10^{-9}$	$10^{-10}$	$10^{-11}$	$10^{-12}$
CV4-35.....	++	++	++	++		
CV4-7.....	++	++	++	++	+-	+-
CV4-8.....	++	++	++	++	++	++
CV4-15.....	++	++	++	++	--	--
CV4-17.....	++	++	++	++	++	-+

activity tests made at different times and under different conditions, it seems likely that preparations of cucumber virus 4 obtained by the milder physical methods retain a greater degree of activity than chemically purified preparations.

*Elementary and Carbohydrate Analyses*—Virus preparations obtained by differential centrifugation were dialyzed against distilled water for 48 hours, frozen, and dried *in vacuo*, and then further dried to constant weight at  $110^{\circ}$  in a drying oven or *in vacuo* over phosphorus pentoxide. The white, fluffy material thus obtained was used for analysis. It should be mentioned that the hygroscopic and voluminous nature of the dry virus and its tendency to become electrically charged make it difficult to weigh out samples with the precision which is common in the cases of many other



proteins. All of the analyses, with the exception of those for carbohydrate and phosphorus, were made by Dr. A. Elek with the customary micromethods. Carbohydrate was determined by the method of Tillmans (11), and most of the phosphorus values (a few were those of Dr. Elek) were obtained with the King colorimetric procedure (12). A Klett-Summerson photoelectric colorimeter was used in both cases. Green Filter 54 and red Filter 66 were used in the colorimeter for carbohydrate and phosphorus analyses, respectively.

A comparison of the analytical data presented in Table II with the values obtained by Bawden and Pirie for cucumber viruses 3 and 4 (2), or with those recorded for tobacco mosaic virus, reveals

TABLE II  
*Chemical Analysis of Cucumber Virus 4*

	No. of prepara- tions	No. of analyses	Mean	Standard deviation of mean
			<i>per cent</i>	<i>per cent</i>
Carbon.....	3	8	50.70	0.16
Hydrogen.....	3	8	6.98	0.06
Nitrogen.....	3	8	15.37	0.15
Sulfur.....	3	8	0.84	0.03
Phosphorus.....	7	12	0.54	0.009
Ash.....	3	8	2.28	0.07
Carbohydrate.....	5	15	2.27	0.10

striking dissimilarities. Following a careful study, only 0.2 per cent sulfur has been found in dialyzed tobacco mosaic virus (13), whereas from 0 to 0.6 per cent sulfur has been reported by Bawden and Pirie for chemically purified cucumber virus 4 (2). In contrast to the latter values, a sulfur content of 0.8 to 0.9 per cent has been found for cucumber virus 4 by repeated analyses on three preparations obtained by physical means as described above. Also, analyses made on electrodialyzed preparations have indicated that most, if not all, of the sulfur is inseparable from the virus. Hence, it may be concluded that cucumber virus 4 contains a definitely greater amount of sulfur than tobacco mosaic virus.

Of still greater significance in a comparative study of these nucleoproteins is the previously unreported fact that the sulfur

in cucumber virus 4 differs decidedly in nature from that of tobacco mosaic virus. Whereas even mildly denatured tobacco mosaic virus gives a strongly positive nitroprusside test, cucumber virus 4, with its larger amount of sulfur, gives a negative test even under conditions which presumably convert disulfide linkages to sulfhydryl groups. This suggests that cucumber virus 4 contains neither the customary sulfhydryl nor disulfide sulfur. A further investigation of the nature of the sulfur in cucumber virus 4 is in progress and will be reported in a later paper.

*Nucleic Acid*.—Nucleic acid was separated from the protein portion of the virus by the method of Johnson and Harkins (14). A greater difficulty in effecting this separation than in the cases of certain strains of tobacco mosaic virus may indicate that there is a stronger bond between protein and nucleic acid in cucumber virus 4.

The nucleic acid, in the form of a dry white powder, gave a negative biuret test and was shown by analysis to contain 34.45 per cent C, 3.80 per cent H, 15.35 per cent N, and 8.83 per cent P. It gave a strongly positive Bial test for pentose (15) but failed to react with diphenylamine in acetic acid (16), which indicates that nucleic acid of the desoxypentose type was absent. From these tests it may be concluded that the nucleic acid of cucumber virus 4, like that of tobacco mosaic virus, is of the yeast type.

*Ultracentrifugation of Virus*.—Sedimentation constants for the virus at two dilutions were kindly determined by Dr. Max A. Lauffer. At concentrations of 4.0 and 0.9 mg. of virus per ml., values of  $173 \times 10^{-13}$  and  $183 \times 10^{-13}$ , respectively, were obtained. These values do not differ significantly from those obtained for tobacco mosaic virus at corresponding dilutions (17). This fact, when considered with x-ray (3, 18) and electron microscope data (19), indicates that the molecular weights of cucumber virus 4 and tobacco mosaic are substantially the same. A value of about  $4 \times 10^7$  has been assigned to the latter (20).

*Electron Micrographs*.—Solutions of cucumber virus 4 exhibit stream double refraction and, upon standing, concentrated solutions of the virus may separate into two layers, of which the lower more concentrated one is liquid crystalline (2). These facts have been confirmed in the present study. In addition, it has been shown that, when a solution of the virus is caused to flow,

the entire stream is doubly refracting, and the double refraction persists for a time after the stream leaves a pipette. These results indicate that the particles of cucumber virus 4, like those of tobacco mosaic virus, are rod-shaped. X-ray measurements (18) and electron micrographs (19) are in complete accord with this conclusion.

A marked tendency towards an end-to-end aggregation of particles was noted in the first electron microscope pictures of cucumber virus 4 (19). As a result, it was possible to measure the



FIG. 1. An electron micrograph of cucumber virus 4 showing typical rod-shaped particles.  $\times 20,000$ .

lengths of only a few individual particles and a value of about  $300\text{ m}\mu$  was reported. In more recent micrographs, such as are reproduced in Fig. 1, less aggregation has been observed. Measurements on the newer micrographs have indicated a particle length of about  $275\text{ m}\mu$  in a considerable number of cases. This value agrees very closely with the figure of  $280\text{ m}\mu$  assigned to tobacco mosaic virus (19). The diameter of the rods, as was expected from x-ray data and the previous electron micrographs, was found to be about the same as that of tobacco mosaic virus; *i.e.*, in the neighborhood of  $15\text{ m}\mu$ . It should be mentioned that x-ray data

indicate a particle thickness of  $14.6\text{ m}\mu$  for cucumber viruses 3 and 4, a value considered to be significantly smaller than the  $15\text{ m}\mu$  for tobacco mosaic virus (3, 18).

A number of rods with shorter lengths than that corresponding to  $275\text{ m}\mu$  are conspicuous in Fig. 1 and similar micrographs. Short particles of this type have also appeared in micrographs of tobacco mosaic virus (19). Since the virus preparations represented in such pictures have always been at least a few days old, it is possible that the short particles of variable length may indicate an increased susceptibility of the rods to fragmentation as the virus ages. If this were the case, the technique of preparing the mounts for the microscope (19) might aid in the rupture of rods of a standard length to produce the observed short particles.

In any event, it may be concluded that electron micrographs, like the data obtained by means of the analytical ultracentrifuge and by x-ray studies, show that the particles of cucumber virus 4 are essentially the same size and shape as those of tobacco mosaic virus.

It is a pleasure to acknowledge indebtedness to Dr. T. F. Anderson, RCA Fellow of the National Research Council, for the micrographs referred to above. The electron microscope used was one generously made available by the RCA Manufacturing Company, Inc., at Camden.

*Serological Tests*—Cucumber virus 4 antiserum was obtained from the blood of a rabbit 8 to 10 days after the last of five spaced intravenous injections of a total of about 40 mg. of virus. Precipitin tests were made by adding 0.3 ml. of antigen at various dilutions to tubes containing 0.3 ml. of cucumber virus antiserum diluted 1:10. All dilutions were made with 0.85 per cent sodium chloride. After mixing, the tubes were incubated at  $37^\circ$  for 2 hours, placed in a refrigerator overnight, and examined for precipitates.

Precipitin tests with cucumber viruses 3 and 4 and six strains of tobacco mosaic virus as antigens and cucumber virus 4 antiserum demonstrated a strong serological relationship between cucumber viruses 4 and 3, but only a feeble relationship between cucumber virus 4 and the six strains of tobacco mosaic virus listed in Table III.

TABLE III

*Precipitation of Cucumber Viruses 3 and 4 and Strains of Tobacco Mosaic Virus with Cucumber Virus 4 Antiserum*

The signs indicate the degree of precipitation.

Antigen	Dilution of antigen, 1:1 = 1 mg. per ml.					
	1:1	1:4	1:16	1:64	1:256	1:1024
Cucumber virus 4.....	++++	++++	+++	++	++	+
"    "    3.....	++++	++++	+++	++	++	+
Tobacco mosaic virus.....	—	±	+	+	+	—
Yellow aucuba mosaic virus..	—	±	+	+	+	±
Green    "    "    ".....	—	±	±	+	+	±
Holmes' masked virus.....	—	±	±	+	+	+
"    ribgrass    ".....	—	—	±	+	+	+
J14D1 virus.....	—	±	+	+	+	—

## DISCUSSION

The activity of physically purified cucumber virus 4 was found in the present study to be definitely higher than that reported by Bawden and Pirie for virus purified by chemical methods. The fact that the latter investigators reported amounts of sulfur ranging from 0.0 to 0.6 per cent strongly suggests that their material contained appreciable amounts of altered virus, for in the present investigation virus purified by mild means has been found to contain 0.84 per cent sulfur.

The presence of about four times as much sulfur in cucumber virus 4 as in tobacco mosaic virus is of special interest. This sulfur is apparently an integral part of the virus, for, as will be described in detail in a later communication, it is not removed even by electro-dialysis. Equally significant is the fact that among ten plant viruses tested in this laboratory only tobacco ring spot (9) and cucumber virus 4 gave a negative nitroprusside test. Thus, the sulfur of the latter virus differs both quantitatively and qualitatively from that of tobacco mosaic virus to which it is presumably related. It seems quite possible that elucidation of the nature of the sulfur in cucumber virus 4 may help to explain biological and immunological differences between this nucleoprotein and those of the tobacco mosaic virus group. It may also have an important bearing on the problem of the narrow host range of the cucumber virus.

The relationship of cucumber viruses 3 and 4 to tobacco mosaic virus is based to some extent on the results of serological tests made

by Bawden and Pirie (2). In our hands, such tests have indicated an even weaker relationship than that previously reported. As will be shown in other communications from this laboratory, there is considerable chemical evidence to account for the feeble precipitin tests which we have observed (21).

The present study has shown that cucumber virus 4 closely resembles tobacco mosaic virus in many respects, but that the two viruses differ fundamentally in their serological behavior and in the nature and amount of the sulfur which they contain.

#### SUMMARY

Cucumber virus 4 has been isolated from diseased cucumber plants and purified by differential centrifugation. With two exceptions, the general properties of the virus obtained by this method were essentially the same as reported by Bawden and Pirie for virus purified by chemical methods. The activity of the present preparations was found to be somewhat greater than that previously reported for chemically isolated virus. Analyses made on three samples of dry virus indicated an average sulfur content of 0.84 per cent, which is about 4 times the amount found in tobacco mosaic virus and is significantly higher than values previously reported by Bawden and Pirie for cucumber virus 4. In contrast to a number of other plant viruses, denatured cucumber virus 4 gave a negative nitroprusside test for S—S and S—H.

Nucleic acid was isolated from the virus and found, like that of tobacco mosaic virus, to be of the yeast type.

Data obtained by means of the analytical ultracentrifuge and the electron microscope showed that the particles of cucumber virus 4, like those of tobacco mosaic virus, were rod-shaped, had a diameter of about 15 m $\mu$ , a length of 275 m $\mu$ , and a molecular weight in the neighborhood of  $4 \times 10^7$ .

Precipitin tests with cucumber viruses 3 and 4 and six strains of tobacco mosaic virus as antigens and cucumber virus 4 antiserum indicated a strong serological relationship between cucumber viruses 3 and 4, but only a weak relationship between the latter and strains of the tobacco mosaic group.

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# AROMATIC AMINO ACIDS IN STRAINS OF TOBACCO MOSAIC VIRUS AND IN THE RELATED CUCUMBER VIRUSES 3 AND 4\*

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One of the striking properties of the high molecular weight nucleoproteins designated as viruses is their ability to reproduce themselves within certain living cells. Equally remarkable is the frequent sudden variation in some viruses by means of which a new strain arises possessing properties quite distinct from those of the original virus. Such a change presumably closely resembles a gene mutation in that the transformation involves nucleoprotein and may be a permanent one which, as reflected by the constancy of accompanying new properties, is faithfully reproduced in subsequent generations of the virus.

It is obvious that the mutation of either a virus or a gene must be attended by chemical changes in structure. Recently, mutations of tobacco mosaic virus induced by x-rays and  $\gamma$ -rays have been reported (1). On the basis of a 15 per cent difference between the phosphorus contents of the original strain and one of the altered strains, presumably established by only two analyses in each case, it was concluded that such mutations could be attributed to irradiation-induced alterations in the nucleic acid part of the virus molecule. In contrast to these findings, data are given in the present paper which show that, in the case of spontaneously arising strains of tobacco mosaic virus, the chemical differences between strains probably lies not in the nucleic acid but rather in the protein part of the virus molecule.

Preliminary to a more extensive investigation, analyses were

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made for tyrosine, tryptophane, and phenylalanine in six well characterized and distinctive strains of tobacco mosaic virus (Fig. 1) and in the related cucumber viruses 3 and 4. The choice of the aromatic amino acids for an approach to this study was prompted by the close association of biological activity in certain enzymes

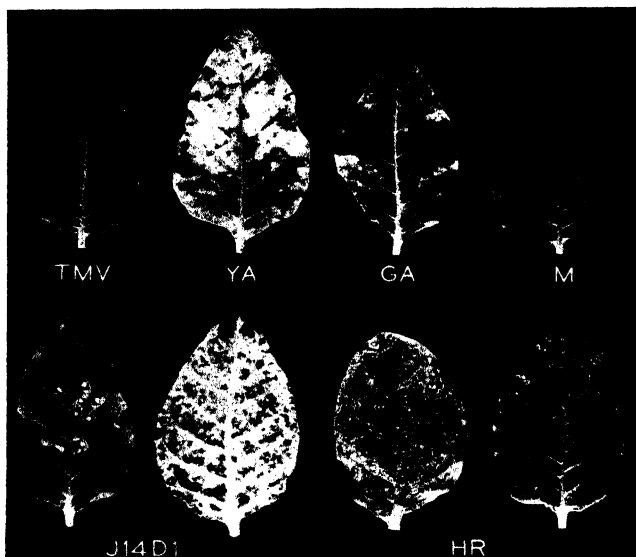


FIG. 1. Leaves of Turkish tobacco plants showing symptoms typical for each of six strains of tobacco mosaic virus. *TMV* = tobacco mosaic virus; *YA* = yellow aucuba; *GA* = green aucuba; *M* = Holmes' masked strain; *J14D1* = a derivative, isolated by Dr. L. O. Kunkel, of Dr. J. H. Jensen's *J14* virus; and *HR* = Holmes' ribgrass strain. The leaf showing the masked strain (*M*) is practically indistinguishable from a normal leaf, although it contains an appreciable amount of virus. The *J14D1* and *HR* viruses differ from the other strains shown in giving distinct primary lesions on the inoculated leaves as well as typical secondary symptoms. In each of these cases, the first leaf shows characteristic primary lesions and the second leaf the secondary symptoms. (Photograph by J. A. Carlile.)

and hormones with the integrity of tyrosine (2-5). Moreover, evidence has accumulated which emphasizes the importance of aromatic nuclei in serological specificity (6). Since relationships between viruses are partly established by serological tests, any chemical evidence which can be correlated with such tests becomes especially desirable.

## EXPERIMENTAL

*Amino Acid Analyses*—Purified virus preparations obtained from filtered infectious juices by differential centrifugation were dialyzed against flowing distilled water for 48 hours, frozen, and dried *in vacuo*, and then further dried to constant weight at 110° in a drying oven. The white fluffy material thus obtained was used for amino acid and phosphorus analyses.

For the tyrosine and phenylalanine determinations, 20 to 40 mg. of virus were hydrolyzed in 0.4 to 0.8 ml. of 6 N sodium hydroxide by heating in a sealed tube in a boiling water bath for 5 hours. The hydrolysate was transferred quantitatively to a 25 ml. volumetric flask and made to volume with distilled water. Aliquots of such solutions were used for the color reactions described below. Although most of the tryptophane analyses reported here were made on alkaline solutions of the dry virus, rather than on hydrolysates, it was found possible to make these determinations satisfactorily on aliquots of the same hydrolysate used for tyrosine and phenylalanine. Thus, it was possible to make duplicate analyses for each of the aromatic amino acids from an original sample of as little as 20 mg. of virus.

Tyrosine was determined by the Bernhart micromethod (7), tryptophane by the Shaw and McFarlane glyoxylic acid procedure (8), and phenylalanine by Block's modification of the Kapeller-Adler reaction (9, 10). The colors developed in each case were compared with appropriate standards in a Klett-Summerson photoelectric colorimeter. Green Filter 54 was used in the tyrosine determination and a combination of Wratten Filters N61 and Flavazine T16 (wave-length, 560 m $\mu$ ) was employed for the tryptophane and phenylalanine analyses. The results of individual analyses showed a maximum deviation from the averages listed in Table I of  $\pm 0.1$  per cent for the tyrosine and  $\pm 0.2$  per cent for the tryptophane and phenylalanine values.

In the phenylalanine determination, it was found necessary to correct for interference by tryptophane. When values for phenylalanine which seemed erroneously high were obtained, an investigation was made with the results summarized graphically in Fig. 2. On the basis of the size of the virus samples used for phenylalanine determinations and the reported amino acid composition of tobacco mosaic virus (11, 12), an amino acid mixture was prepared with only the aromatic amino acids omitted. When

various amounts of this mixture or any of the known constituents of tobacco mosaic virus were added to a phenylalanine standard, no change in the colorimeter reading was observed except when tryptophane was included. Upon addition of tryptophane, the reading of the standard became higher in proportion to the amount

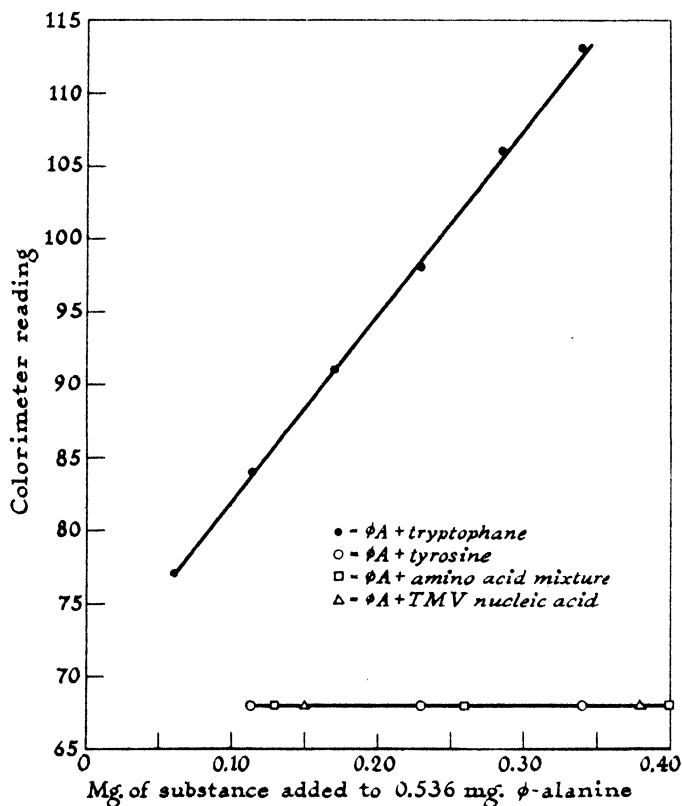


FIG. 2. Effect of different materials on the colorimetric determination of phenylalanine ( $\phi$ -alanine,  $\phi A$ ). *TMV* represents tobacco mosaic virus.

of tryptophane added. Consequently, in analyses of virus for phenylalanine, a value corresponding to the amount of tryptophane present in the aliquot of solution taken for analysis was subtracted from the colorimeter reading of the unknown before the calculations were made. The validity and the basic numerical

value of such a correction were established by a series of determinations in which different amounts of tryptophane were added to a constant amount of phenylalanine and the reverse. In all cases, a constant increment of color was observed for a definite amount of tryptophane. In our experience, the chief advantages of the above procedure as opposed to the method applied to acid hydrolysates (9), in which tryptophane has been automatically destroyed, are convenience, greater reproducibility, smaller

TABLE I  
*Aromatic Amino Acids and Phosphorus in Strains of Tobacco Mosaic Virus  
and in Cucumber Viruses 3 and 4*

Virus	No. of prepara- tions*	Tyrosine	Trypto- phane	Phenyl- alanine	P†
		per cent	per cent	per cent	per cent
Tobacco mosaic.....	12	3.8	4.5	6.0	0.56
Yellow aucuba.....	3	3.9	4.2	6.3	0.52
Green ".....	2	3.9	4.2	6.1	0.54
Holmes' ribgrass.....	4	6.4	3.5	4.3	0.53
" masked.....	2	3.9	4.3	6.1	0.54
J14D1.....	2	3.8	4.4	6.1	0.55
Cucumber virus 4.....	7	3.8	1.4	10.2	0.54
" " 3.....	1	4.0	1.5	10.0	0.56

\* Two or more analyses were made on each preparation. The results of individual analyses showed a maximum deviation from the averages listed of  $\pm 0.1$  per cent for the tyrosine and  $\pm 0.2$  per cent for the tryptophane and phenylalanine values.

† The values in this column represent the average of duplicate determinations on two or more preparations, with the exception of cucumber virus 3. Almost all of the results of individual analyses were within the range 0.52 to 0.58 per cent.

hydrolytic losses of phenylalanine (10), and avoidance of mechanical losses involved in decolorization of acid hydrolysates. The main disadvantage appears to be the necessity of knowing the tryptophane content of the protein in order to estimate the phenylalanine content.

Analysis of twelve preparations of tobacco mosaic virus indicated the presence of 3.8, 4.5, and 6.0 per cent of tyrosine, tryptophane, and phenylalanine, respectively. These values agree quite well with those recently reported for tobacco mosaic virus

(11). The results obtained for yellow aucuba, green aucuba, Holmes' masked, and J14D1 strains of tobacco mosaic virus were similar. However, pronounced differences were found in the cases of Holmes' ribgrass strain and cucumber viruses 3 and 4. Tyrosine, tryptophane, and phenylalanine contents of 6.4, 3.5, and 4.3 per cent, respectively, were found for the ribgrass strain, while similar analyses of seven preparations of cucumber virus 4 and one preparation of cucumber virus 3 indicated the presence of about 3.8, 1.4, and 10.2 per cent of tyrosine, tryptophane, and phenylalanine, respectively.

*Phosphorus Analyses*—Phosphorus determinations were made on 5 to 10 mg. samples of dried virus according to the method of King (13). Red Filter 66 was used in the colorimeter.

Individual analyses for phosphorus in samples of the various strains resulted in values ranging from 0.5 to 0.6 per cent, with a majority of the values falling within a somewhat narrower range. While such a variance would be considered serious in the analysis of many materials, it is perhaps not so in the case of viruses for two reasons. The first of these is that the phosphorus of viruses is contained entirely in the most labile portion of the virus molecule, *i.e.* in the nucleic acid, and is, therefore, most subject to loss during purification of the virus. Second, and possibly of much greater importance, is the fact that the hygroscopic and voluminous nature of the dry virus and its tendency to become electrically charged make it difficult to weigh out samples with the precision which is common in the cases of many other proteins. Hence, the differences between the average phosphorus value for tobacco mosaic virus and the phosphorus values for any of the strains listed in Table I are smaller in many cases than the differences between individual samples of tobacco mosaic virus or even between values obtained during repeated analyses of the same sample. From this fact, it may be concluded that there is no demonstrable quantitative difference between the nucleic acid components of the viruses examined. This does not exclude the possibility of qualitative dissimilarities among the nucleic acid constituents. However, such a contingency is less likely than it at first appears, since the positive Bial and negative Dische tests which were obtained for each virus indicate that all of them, as has been definitely shown for tobacco mosaic virus (14), contain the yeast type of nucleic acid.

*Serological Reactions*—Preparations of the viruses used in serological tests were made by purely physical methods involving filtration and differential centrifugation of the juices from infected Turkish tobacco plants, or from cucumber plants in the cases of cucumber viruses 3 and 4. Tobacco mosaic virus antiserum was obtained from the blood of a rabbit 8 to 10 days after the last of five spaced intravenous injections of a total of about 40 mg. of virus. Precipitin tests were made by adding 0.3 ml. of antigen at various dilutions to tubes containing 0.3 ml. of tobacco mosaic virus antiserum diluted 1:10. All dilutions were made with 0.85 per cent sodium chloride. After mixing, the tubes were incubated

TABLE II

*Precipitation of Strains of Tobacco Mosaic Virus and Cucumber Viruses 3 and 4 with Tobacco Mosaic Virus Antiserum*

The signs indicate the degree of precipitation.

Antigen	Dilution of antigen, 1:1 = 1 mg. per ml.					
	1:1	1:4	1:16	1:64	1:256	1:1024
Tobacco mosaic.....	++++	++++	+++	++	+	±
Yellow aucuba.....	++++	++++	+++	++	+	—
Green ".....	++++	++++	+++	++	+	—
Holmes' masked.....	++++	++++	+++	++	+	—
✓ " ribgrass.....	—	+	++	+	—	—
J14D1.....	++++	++++	+++	++	+	—
Cucumber virus 4.....	—	+	+	+	—	—
" " 3.....	—	+	+	+	—	—

at 37° for 2 hours, placed in a refrigerator overnight, and examined for precipitates.

Precipitin tests with strains of tobacco mosaic virus and cucumber viruses 3 and 4 as antigens and tobacco mosaic virus antiserum demonstrated a strong serological relationship between tobacco mosaic virus and all of the viruses tested, with the exception of Holmes' ribgrass strain and cucumber viruses 3 and 4 (see Table II).

A comparison of the amino acid values for the viruses examined (Table I) with the results of the serological tests reveals a striking similarity in the outcome of the two types of experiments. Within the limits of accuracy of the methods employed, the aromatic amino acid compositions of the viruses examined were very close

to that of tobacco mosaic virus, with the exception of Holmes' ribgrass strain of tobacco mosaic virus and cucumber viruses 3 and 4. In each of the latter cases pronounced differences from the composition of tobacco mosaic virus were apparent. It may or may not be significant that in all cases the total percentages of aromatic amino acids were about the same.

It is of some interest to note in passing that precipitates, comparable to those obtained with rabbit antiserum, were also observed in precipitin tests with tobacco mosaic virus antiserum obtained from a mangabey monkey. The authors are indebted to Dr. K. W. Thompson of Yale University for the latter antiserum.

#### DISCUSSION

Most important among the characteristics by which plant virus strains are recognized are the capacity of one strain to protect a fully infected plant from further infection by a second strain and the ability of one strain to react with the antiserum of another. In addition, viruses shown to be related by plant protection and serological tests invariably possess similar physicochemical properties and are transmitted by the same methods. By these criteria, the viruses discussed in the present paper are strains of tobacco mosaic virus. They are known to possess very similar physicochemical properties, are transmitted in the same manner, react with each other's antiserum, and plants fully infected with one are protected against infection from another. In the cases of cucumber viruses 3 and 4, it has not been possible to apply the plant protection test, for no host common to the cucumber viruses and tobacco mosaic virus has thus far been found. The cucumber viruses are somewhat unusual in that they have been found to multiply only in members of the Cucurbitaceae. Despite this fact, it is commonly believed that cucumber viruses 3 and 4 may have arisen from tobacco mosaic virus through some fortuitous event. In this connection it is important to note that these nucleoproteins have very similar general properties (15, 16), and especially that among a large number of viruses cucumber viruses 3 and 4, tobacco necrosis virus, and the viruses of the tobacco mosaic group stand alone in their remarkable ability to resist heat. Therefore, the pronounced differences in the amounts of aromatic amino acids found in tobacco mosaic virus, Holmes'

ribgrass strain, and cucumber viruses 3 and 4 demonstrate clearly for the first time the chemical nature of certain changes which may accompany the variation of a virus. The differences in composition of tobacco mosaic virus and the Holmes' ribgrass virus are particularly important, for the latter is unequivocally a strain of tobacco mosaic virus (17). In this case at least, the formation of a virus variant obviously has involved fundamental changes in the composition of the protein rather than simpler alterations of the type frequently postulated, such as a gain or loss of certain reactive groups or a rearrangement of basic units to form a new pattern. For this reason, it seems quite likely that investigations now in progress will reveal distinctive differences in the composition of the proteins of tobacco mosaic virus and the strains which did not differ significantly from the former with respect to aromatic amino acids.

In view of the marked changes which the present data have shown to occur in the variation of a virus, it seems likely that a new strain has arisen in each case by a diversion of the synthetic process by which the virus multiplies rather than by an alteration of some of the completely formed virus molecules. Further evidence in this direction may be provided by the results of certain irradiation experiments (1, 18). Although irradiation of tobacco mosaic virus *in vitro* with x-rays or  $\gamma$ -rays failed to produce variants, the irradiation of diseased plants, or even irradiation of normal plants 24 hours before inoculation, appeared to induce the formation of a small number of variants. The latter results have led to the hypothesis that irradiation somehow alters certain plant constituents with the result that their incorporation into a virus molecule produces a new strain. However, no specific chemical differences between strains isolated following irradiation and the original virus have been demonstrated, with the exception of a 15 per cent difference between the phosphorus values for two strains (1). Data obtained in the present investigation indicate tyrosine, tryptophane, and phenylalanine contents of 6.4, 3.5, and 4.3 per cent, respectively, for one spontaneously occurring strain of tobacco mosaic virus. These values vary greatly from the corresponding ones of 3.8, 4.5, and 6.0 per cent established for ordinary tobacco mosaic virus and show that a profound difference exists in the composition of the protein components of the two



virus strains. Because of the deep seated nature of this difference, it is suggested that mutation, or the formation of a virus variant, occurs during the process of virus synthesis.

The remarkable coincidence of results of the serological tests and the amino acid analyses is of special interest. Extensive work will be required to establish fully the significance of this agreement, but, when considered with evidence of other types pointing in the same direction (6), it appears to reemphasize the rôle of aromatic amino acids in the serological specificity of proteins.

The authors wish to express their appreciation to Dr. F. O. Holmes for making a portion of this study possible by kindly providing the ribgrass strain of tobacco mosaic virus.

#### SUMMARY

Serological tests with six strains of tobacco mosaic virus and cucumber viruses 3 and 4 as antigens and tobacco mosaic virus antiserum showed a close relationship between tobacco mosaic virus and all of the viruses tested, with the exception of Holmes' ribgrass strain and cucumber viruses 3 and 4.

Analysis of twelve preparations of tobacco mosaic virus indicated the presence of 3.8, 4.5, and 6.0 per cent of tyrosine, tryptophane, and phenylalanine, respectively. The results obtained for yellow aucuba, green aucuba, Holmes' masked, and J14D1 strains of tobacco mosaic virus were the same as those for tobacco mosaic virus within experimental error. Pronounced differences were found in the cases of Holmes' ribgrass strain of tobacco mosaic virus and cucumber viruses 3 and 4, for the tyrosine, tryptophane, and phenylalanine values for the former were 6.4, 3.5, and 4.3 per cent, respectively, and about 3.8, 1.4, and 10.2 per cent, respectively, for the cucumber viruses.

An indication of the absence of significant quantitative differences in the nucleic acid component of the viruses was evident in the close agreement found in analytical values for phosphorus in the various strains. Qualitative tests showed that the nucleic acid in all of the viruses was of the yeast type.

On the other hand, the decided dissimilarities in the protein portions of some of the viruses, as revealed by the results of amino acid analyses, show that the mutation of tobacco mosaic virus to

form a new strain may be accompanied by changes in the amino acid composition of the virus. Because of the nature of these changes, it is suggested that a new virus strain arises by a diversion of the synthetic process by means of which the virus multiplies rather than by an alteration of completely formed virus molecules.

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## THE VERATRINE ALKALOIDS

### XI. THE DEHYDROGENATION OF JERVINE

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Since the first investigation of the alkaloids contained in the three liliaceous plants, *Sabadilla officinalis* (*Veratrum sabadilla*), *Veratrum album*, and *Veratrum viride*, as well as several allied species of the veratrums, it has been customary to group them together and to consider the possibility that they are chemically related. Following the older work of Wright and Luff (1) and of Salzberger (2), who made important contributions in regard to the occurrence and formulations of a number of these alkaloids, the recent careful studies of Poethka (3) on the isolation and distribution of the alkaloids in *V. album* have added the alkaloid germerine to the list and also contributed to our knowledge of the formulations and superficial chemistry of a number of them. It appears definite from what has been done that they belong in two separate categories. The first consists of those which do not behave as ester alkaloids; viz., jervine,  $C_{26}H_{37}O_3N$ , rubijervine,  $C_{26}H_{43}O_2N$ , and pseudojervine,  $C_{33}H_{49}O_8N$  ( $C_{29}H_{43}O_7N$ , according to Wright and Luff). None of these appears to have been found in *sabadilla* seeds. The second category is that of the highly toxic alkaloids of *sabadilla* seeds, cevadine and veratridine (4), which are respectively the angelic (or tiglic) and veratric esters of cevine,  $C_{27}H_{43}O_8N$ , and protoveratrine and germerine which were isolated from *V. album*. The protoveratrine of Salzberger, according to Poethke, possesses the formulation  $C_{40}H_{63}O_{14}N$  and yields on saponification the three acids, acetic, *l*-methylethylacetic, and methylethylglycolic acids and the amorphous alkamine protoverine,  $C_{28}H_{45}O_{10}N$ . Germerine is the methylethylacetic and methylethylglycolic esters of a -crystalline alkamine germine,  $C_{26}H_{41}O_8N$ .

The close resemblance of the formulation of these alkamines supports the view of a close chemical relationship. However, this has not as yet been supported by any evidence obtained by comparative degradative investigations.

In connection with our own study of the degradation of cevine, we have turned to a number of these alkaloids with the hope not only of confirming the possibility of a mutual relationship but that certain of the alkaloids in the first category with fewer oxygen atoms would lend themselves more readily to degradative study. We wish here to report our experience with jervine.

According to Saito, Suginome, and Takaoka (5), this alkaloid is a secondary amine which also possesses an acylatable hydroxyl group and contains 2 active H atoms. These observations were confirmed by Poethke who, however, has concluded that the remaining 2 oxygen atoms are not contained in a methylenedioxy group.

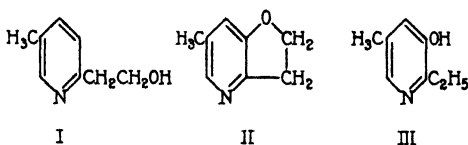
On distillation with soda lime, jervine was found to behave quite differently from cevine in our experience. Only a relatively small amount of a volatile basic fraction was produced which after hydrogenation was fractionated. This resulted in a very small yield of a crystalline oxygen-containing base, analysis of which suggested the formulation  $C_8H_{17}ON$ . (After recrystallization from ether it melted not sharply at 100–115°. Because of the very small amount of material available, it was analyzed without further recrystallization.  $C_8H_{17}ON$ , calculated, C 67.07, H 11.97, N 9.79; found, C 66.97, H 11.81, N 9.43.) It is possibly related to the base  $C_8H_{11}ON$  obtained by dehydrogenation of jervine as described below. Most of the nitrogen-containing material was retained in the soda lime. This will be a subject of future study.

The dehydrogenation of jervine with selenium gave more promising results and produced a mixture which was separated into basic and neutral fractions from which a number of substances have been isolated by careful manipulation.

From the more volatile basic fraction a weak base was obtained which after purification as the picrate appeared on comparison to be identical with the picrate of 2-ethyl-5-methylpyridine obtained from cevine (6). In addition a phenolic basic fraction was separated, from which an appreciable yield of a crystalline substance was isolated which melted at 145–147°. Analysis supported the

formulation  $C_8H_{11}ON$  or that of a hydroxyethylmethylpyridine. The base coupled with diazotized sulfanilic acid. The identity of this substance remains to be established but its probable identity may be inferred from the following considerations.

In our earlier work on the degradation of cevine by different procedures two oxygen-containing bases were obtained among others; *viz.*,  $C_8H_{11}ON$  and  $C_8H_9ON$ . The former was shown to be a derivative of 2-ethyl-5-methylpyridine containing a hydroxyl group on one of the side chains, presumably the ethyl (Formula I).



Although not rigidly established, a possible relationship between these bases was tentatively assumed (7), so that it could be considered that the base  $C_8H_9ON$  is the ether of the hydroxy base due to cyclization on position 3 (Formula II). It therefore appears not unlikely that the phenolic base from jervine will prove to be 2-ethyl-5-methyl-3-hydroxypyridine (Formula III).

From the least volatile basic fraction of the dehydrogenation products from jervine which corresponded to the cevanthridine fraction obtained from cevine, no crystalline substance has been as yet isolated and no evidence of the formation of cevanthridine or analogous base has been obtained. The study of this fraction will be resumed as opportunity is presented.

The least volatile neutral fraction has yielded a number of substances, mainly hydrocarbons, after chromatographic separation with alumina. The majority (about three-fourths) of the neutral material passed readily through the column and consisted of a mixture of hydrocarbons. This mixture on careful fractional distillation was separated into the following fractions.

The lowest boiling of these was purified as the picrate, the formulation of which was found on analysis to be  $C_{14}H_{14} \cdot C_6H_3O_7N_3$ . This conclusion was supported by analysis of the hydrocarbon itself which, however, could not be crystallized. The ultraviolet absorption spectrum curve of this substance as represented in Fig. 1

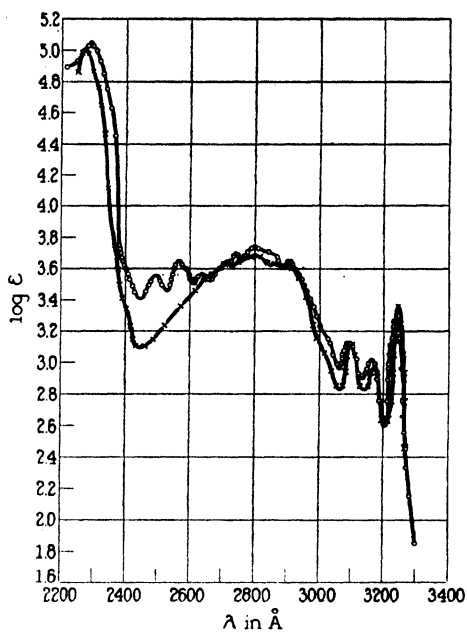
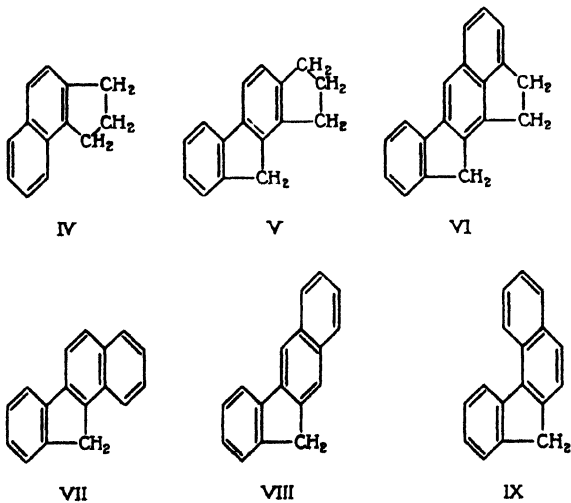


FIG. 1. Absorption spectrum curves.  $\times$  = benzohydrindene,  $\circ$  =  $C_{14}H_{14}$  hydrocarbon.



closely resembled that of 4,5-benzohydrindene (Formula IV). It must be concluded therefore that this hydrocarbon is a methyl-4,5-benzohydrindene homologous with the substance obtained on dehydrogenation of cevine (7, 8).

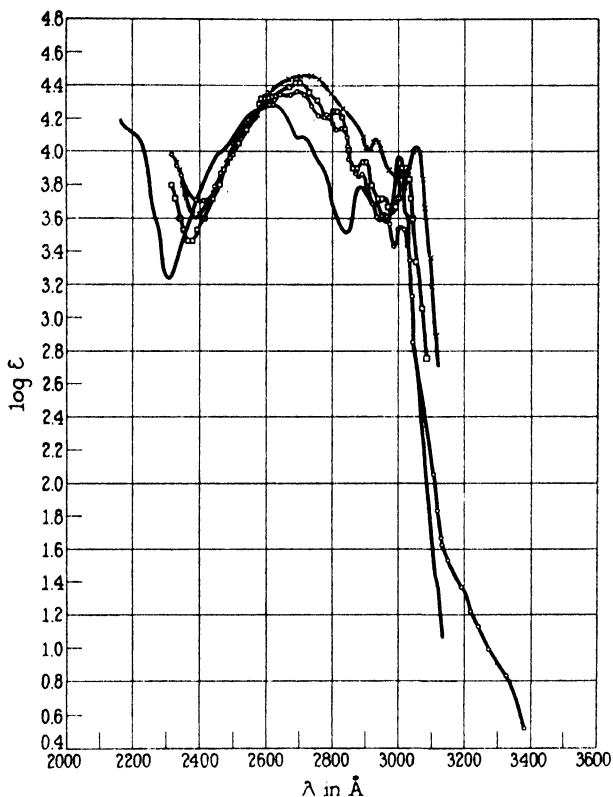


FIG. 2. Absorption spectrum curves.  $\times$  =  $C_{24}H_{30}$  hydrocarbon,  $\circ$  =  $C_{17}H_{16}$  hydrocarbon,  $\square$  =  $C_{20}H_{22}$  hydrocarbon, the solid line = fluorene.

A higher boiling fraction yielded a crystalline hydrocarbon which melted at 79°. The analysis and molecular weight determination supported the formulation  $C_{20}H_{22}$ . The absorption spectrum curve of this substance as shown in Fig. 2 was very similar to that of fluorene.

A later fraction was separated with difficulty into the  $C_{20}H_{16}$  hydrocarbon described below and an amount of a hydrocarbon too



small for final purification. But the analysis and molecular weight determination suggested a homologue of the previous  $C_{20}H_{22}$  hydrocarbon; viz.,  $C_{21}H_{24}$ .

From the fraction which followed that above, a crystalline hydrocarbon was obtained which melted at  $125-127^\circ$ . The analysis and molecular weight determination indicated a formulation

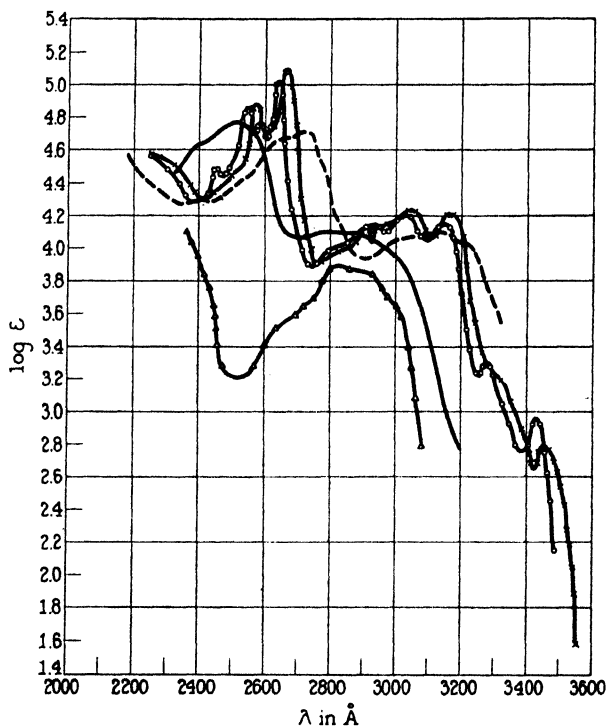


FIG. 3. Absorption spectrum curves.  $\times$  =  $C_{22}H_{20}$  hydrocarbon,  $\circ$  =  $C_{20}H_{16}$  hydrocarbon, the solid line =  $\beta$ -phenylnaphthalene, the dash line = dihydromethylcholanthrene,  $\Delta$  =  $\alpha$ -phenylnaphthalene.

$C_{20}H_{16}$ . The substance did not form a stable picrate. The absorption spectrum curve obtained with this hydrocarbon is presented in Fig. 3.

The next fraction after special chromatographic treatment gave a hydrocarbon,  $C_{24}H_{30}$ . The absorption spectrum of the latter, as shown in Fig. 2, was again very similar to that of fluorene.

Although the melting point of this substance, 100–101°, approached that of the  $C_{24}H_{30}$  hydrocarbon obtained from cevine (8), 106–109°, the different crystalline form as well as the melting point depression obtained showed that they must be isomeric.

Finally, the still higher boiling fraction (No. 12) yielded a crystalline hydrocarbon which melted at 154–155° and analysis and molecular weight determinations were in agreement with the formulation  $C_{22}H_{20}$ . The absorption spectrum curve of this substance in Fig. 3 is similar to that of the above  $C_{20}H_{18}$  hydrocarbon. Investigation of intermediate fractions suggested the presence also of a hydrocarbon  $C_{21}H_{18}$  but this was not obtained with certainty in pure form.

Following the main chromatograph fraction which furnished the above hydrocarbons, later chromatograph fractions were then investigated. These were found to contain but relatively small amounts of material. One of these yielded by fractional distillation a crystalline oxygen-containing substance which melted at 141–145°. Analysis indicated the formulation  $C_{20}H_{22}O$  or that possibly of a simple hydroxyl derivative of the above discussed  $C_{20}H_{22}$  hydrocarbon. The amount of material available was unfortunately too small for an extended study.

The hydrocarbons thus obtained on dehydrogenation of jervine, apart from the simplest  $C_{14}H_{14}$  hydrocarbon, fall into two categories. One of these appears to consist of homologues of a tetracyclic fluorene which may perhaps be represented as a cyclopentenofluorene by Formula V. The second group comprises the hydrocarbons which from the formulations appear to contain an extra aromatic ring and must therefore be regarded as pentacyclic.

As above stated, the  $C_{14}H_{14}$  hydrocarbon must be a methyl homologue of the benzohydrindene obtained from cevine. Although the hydrocarbons  $C_{17}H_{16}$ ,  $C_{18}H_{18}$ ,  $C_{19}H_{20}$ , and  $C_{24}H_{30}$  which were obtained on dehydrogenation of cevine have not been encountered in the degradation of jervine, the isolation of the hydrocarbons  $C_{20}H_{22}$  and  $C_{24}H_{30}$  and possibly  $C_{21}H_{24}$  with absorption curves resembling that given by the cevine hydrocarbon  $C_{17}H_{16}$  as shown in Fig. 2 would seem to leave little doubt of their close relationship. This is further supported by the common Vanscheidt color test given by both series of hydrocarbons.

The hydrocarbons  $C_{20}H_{18}$  and  $C_{22}H_{20}$  from jervine which give a

modified Vanscheidt color test are apparently homologues. This is supported by the close resemblance of their absorption curves (Fig. 3) which are quite different from those of the above cevine-like hydrocarbons. This can be explained by an extra aromatic ring as shown in Formula VI.

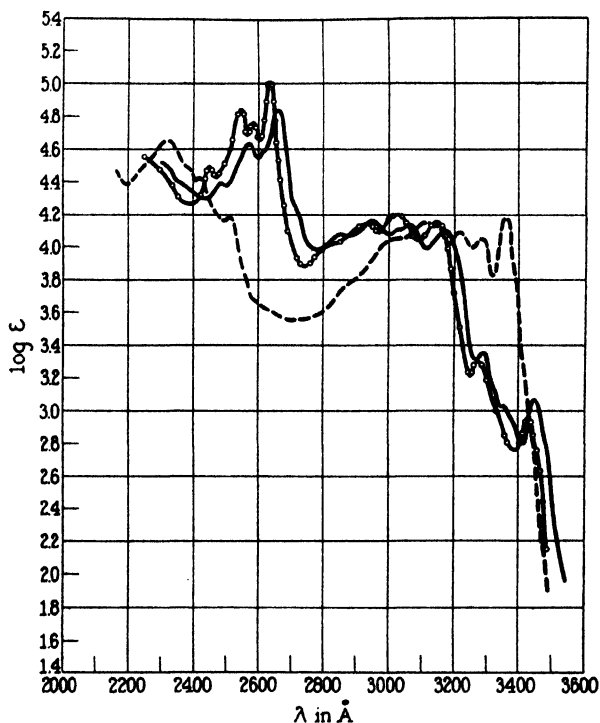


FIG. 4. Absorption spectrum curves.  $\circ$  =  $C_{20}H_{16}$ , the solid line = 1,2-benzofluorene, the dash line = 3,4-benzofluorene.

Such a possibility is strongly supported by the striking similarity in the absorption spectrum curves of 1,2-benzofluorene, Formula VII, as reported by Mayneord and Roe (9). For comparison with the curve of the  $C_{20}H_{16}$  hydrocarbon, we have replotted in Fig. 4 the data given by these workers for this synthetic hydrocarbon as well as for the isomeric 3,4-benzofluorene, Formula IX. It will be seen that in the case of the latter, which is an  $\alpha$ -phenyl-naphthalene derivative, there is considerable divergence of its

curve from that of the jervine hydrocarbon. Unfortunately, we have not been able to find the absorption spectrum curve for the 2,3-benzofluorene, Formula VIII, which, like the 1,2 isomer, could be considered a modified  $\beta$ -phenylnaphthalene. It should be expected, however, that the absorption spectrum curves of these two hydrocarbons should bear a closer resemblance to each other than in the case of the 3,4 isomer. Therefore, there remains a definite possibility that the jervine hydrocarbons  $C_{20}H_{16}$  and  $C_{22}H_{20}$  could be homologues of 2,3-benzofluorene as well as of 1,2-benzofluorene. A further suggestion may be derived from the data presented by Fieser and Hershberg for 6,7-dihydro-20-methylcholanthrene (10) which we have replotted for comparison in Fig. 3 along with the curves of  $\beta$ -phenylnaphthalene and  $\alpha$ -phenylnaphthalene. It will be seen that there is again a general resemblance to the curves of the jervine hydrocarbons except in the case of  $\alpha$ -phenylnaphthalene. It is therefore suggested that in the pentacyclic hydrocarbons from jervine the chromophorically active system is that of a modified  $\beta$ -phenylnaphthalene, as represented in Formula VI.<sup>1</sup>

Such hydrocarbons were not encountered in the study of cevine and it is quite possible that their production from jervine without the formation of cevanthridine-like bases may be due to the different course which the dehydrogenation takes of that portion of the molecule to which the nitrogen is attached in each of these alkaloids. Cevine is a tertiary amine and the nitrogen appears to be common to two of its rings. Jervine reacts as a secondary base and therefore, while apparently closely related to cevine, there must be a difference in the arrangement of that portion of the molecule in the environment of the nitrogen ring. During the dehydrogenation the latter can be ruptured, followed by the formation of a new ring, perhaps bearing a resemblance in principle to the indirect production of methylcholanthrene from desoxycholic acid.

Jervine, according to Saito, Suginome, and Takaoka (5), yields a tetrahydro derivative. This result we have confirmed. It must therefore contain at least two double bonds in the molecule.

<sup>1</sup> A similar type of reasoning has been used by Cook, Hewett, Mayneord, and Roe in their comparative study of the  $C_{25}$  hydrocarbon from cholesterol and synthetic naphthafuorenes (11).

The ultraviolet absorption spectrum of jervine itself is represented in the curve of Fig. 5. The general position of the maximum of 2500 Å. indicates from the experience which has gradually accumulated with other polycyclic substances (12) that the double bonds are conjugated in this alkaloid, and may not be in the same ring but distributed between two adjoining rings. It appears quite certain that jervine does not contain a benzenoid ring.

Since only 1 of the oxygen atoms of the alkaloid has been shown to occur in a hydroxyl group, the remaining 2 oxygen atoms

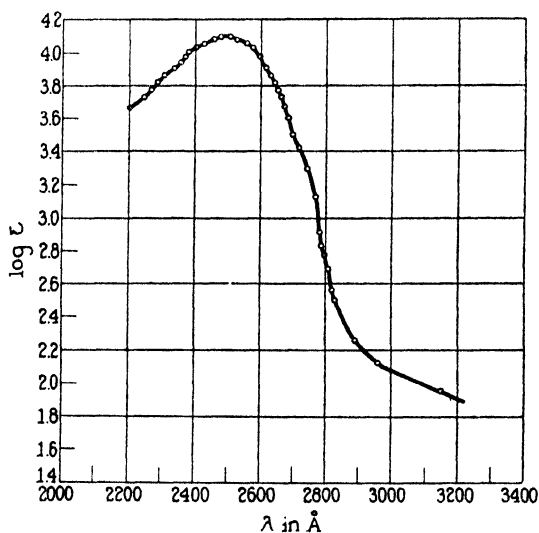


FIG. 5. Absorption spectrum curve for jervine

would appear to be of ether if not of lactone or ketone character. Jervine would thus appear to be at least of hexacyclic if not of heptacyclic character, including the oxygen-containing ring. It is hoped to extend further the comparative study of the degradation of jervine and cevine as well as of other alkaloids of this group.

#### EXPERIMENTAL

*Dehydrogenation of Jervine*—10.8 gm. of jervine were ground with 30 gm. of selenium and the mixture was placed in a 150 cc.

distillation flask. The side arm of the flask was extended somewhat and led into another flask immersed in an ice bath to serve as a condensing trap. The neck of the flask was extended to serve as an air condenser. A glass tube fitted to the top of the flask led to the bottom of the molten mixture and during the dehydrogenation nitrogen gas was slowly passed through the apparatus. The flask was heated in a salt mixture which was raised to a temperature of 340° in approximately 0.5 hour and maintained at that temperature for 2 hours.

After the dehydrogenation, the side arm of the flask was cut off and the contents of the ice trap were washed out with ether and a little water. 2 cc. of 1:1 HCl were added to the mixture and after shaking the ether was removed. Careful examination of the ether extract did not reveal anything promising and it was dis-

TABLE I  
*Fractionation of Volatile Bases*

Fraction No.	Bath temperature	Column temperature	Pressure	Weight of fraction	Micro b.p.	Analysis	
						C	H
	°C.	°C.	mm.	mg.	°C.	per cent	per cent
1	100	68	10	40	164-165	78.10	9.20
2	102	71	3	20	180	.	
3	120	100	0.05	50	222	79.23	9.87
4	180	150	0.05	70		80.21	9.03

carded. The aqueous acid layer was cooled and made alkaline with KOH. Ether extraction of this alkaline solution gave an oily basic residue after drying over  $K_2CO_3$  and concentration. The aqueous alkaline layer was set aside to be treated as described below.

The oily basic residue was placed in a 10 cm. microfractionating column (13) and separated into four fractions (Table I).

The boiling point and carbon figures obtained with Fraction 1 were somewhat low for the 2-ethyl-5-methylpyridine isolated from cevine for which we have recorded the micro boiling point of 171°.

$C_8H_{11}N$ . Calculated, C 79.27, H 9.15; found, C 78.10, H 9.20

However, when the picrate was prepared from this fraction and recrystallized twice from acetone, it formed characteristic leaflets

which melted at 144–145°<sup>2</sup> and showed no melting point depression when mixed with the picrate of 2-ethyl-5-methylpyridine obtained from cevine. It appeared to be identical in every respect.

$C_8H_{11}N \cdot C_6H_3O_7N_3$ . Calculated. C 47.98, H 4.03  
Found. " 48.28, " 3.93

*Phenolic Base*—The above aqueous alkaline layer was saturated with  $CO_2$  and then extracted with ether. The ether extract yielded a residue which was dissolved in a little benzene and then treated with bone-black. The filtrate in turn gave a residue of 0.18 gm. It crystallized from ether in stout square plates which melted at 145–147°. It coupled with diazotized sulfanilic acid to give an orange color on the acid side which changed to a lighter shade when made alkaline. When dissolved in dilute acetic acid, it gave a rather weak red-brown color with ferric chloride.

$C_8H_{11}ON$ . Calculated, C 70.02, H 8.08; found, C 69.99, H 7.92

The residue in the dehydrogenation flask was finely pulverized and well extracted with ether. The ether extract was concentrated to about 100 cc. and then extracted with 50 cc. of 10 per cent HCl. A dark tar precipitated. The ether layer was separated and set aside to be treated for the hydrocarbons as described below. The tar suspended in the acid solution could easily be extracted with chloroform. After extraction with this solvent, practically nothing remained in the acid solution. The chloroform solution was extracted with 10 per cent NaOH and dried over  $K_2CO_3$ . Upon evaporation to dryness a residue of 1.6 gm. was obtained. This corresponds to the cevanthridine fraction in the dehydrogenation of cevine. However, nothing crystallized readily from it and it was set aside to be studied more carefully at some future time.

The ether solution which contained the hydrocarbon fraction was dried and evaporated to dryness. It weighed 4 gm. It was dissolved in 70 cc. of benzene and the solution was passed through a column prepared with 300 gm. of Brockmann's alumina. When material began to emerge with the solvent, 150 cc. were collected. This fraction yielded 3 gm. of partially crystalline material. This

<sup>2</sup> All melting points reported are micro melting points.

was further fractionated by distillation as described below. The next 100 cc. of solvent contained only 200 mg. of oil which was not further investigated. The next 100 cc. contained 100 mg. of material which was partially crystalline. This was dissolved in ether, treated with a little bone-black, and the filtrate was evaporated until crystallization began. 35 mg. of a crystalline substance which melted at 110–140° were collected. A further recrystallization yielded long needles which melted at 141–145°. There was not sufficient material for further examination. The analytical data agreed with those required by a simple oxygen derivative of the  $C_{20}H_{22}$  hydrocarbon described below.

$C_{20}H_{22}O$ . Calculated, C 86.28, H 7.97; found, C 86.38, H 8.08

The above 3 gm. fraction of mixed hydrocarbons was placed in a sublimation apparatus under 0.2 mm. pressure and all was collected which sublimed at a temperature up to 200°. The distillate was then placed in a fractionating apparatus which had a column 22 cm. in length.

The results of the fractionation are given in Table II.

$C_{14}H_{14}$  *Hydrocarbon*—Fraction 1 when treated with 50 mg. of picric acid in alcoholic solution crystallized. After two recrystallizations from alcohol, broad flat orange needles were obtained which melted at 87–89°.

$C_{14}H_{14} \cdot C_6H_5O_7N_3$ . Calculated. C 58.37, H 4.16  
Found. " 58.18, " 3.86

A suspension of the picrate in ether was extracted with 2 per cent NaOH. The colorless ether solution was dried over  $K_2CO_3$  and then concentrated. 22 mg. of an oil were obtained which had a yellow-blue fluorescence. It could not be induced to crystallize and was distilled at about 105° under 0.5 mm. pressure.

$C_{14}H_{14}$ . Calculated, C 92.26, H 7.74; found, C 92.30, H 7.64

The absorption spectrum of this material resembled closely that of 5,6-benzohydrindene.

$C_{20}H_{22}$  *Hydrocarbon*—Fraction 3 was recrystallized directly from ether at –20°. 42 mg. of thin rectangular plates were collected which melted at 73–78°. Two additional recrystallizations did not alter the melting point appreciably and the final material



consisted of needles or rectangles. It appeared to be dimorphic, since it began to melt at 73° but was not entirely molten until 79° was reached. However, when the melt was heated above 79° and then allowed to crystallize, it showed a sharp melting point of 79°.

$C_{20}H_{22}$ . Calculated. C 91.54, H 8.46, mol. wt. 262.18  
 Found. " 91.41, " 8.69, " " 257.6

The molecular weight was determined by the Rast method in camphor.

TABLE II  
*Fractionation of Jervine Hydrocarbons*

Fraction No.	Bath temperature	Column temperature	Pressure	Weight of fraction	
	°C.	°C.	mm.	mg.	
1	190	150	0.5		Oil
2	195	150	0.4		"
3	200	170	0.35		Crystals
4	210	175	0.3		"
5	215	185	0.3	180	"
6	220	185	0.3	180	"
7	220	185	0.3	125	"
8	220	185	0.3	115	"
9	220	185	0.3	115	"
10	220	190	0.3	92	"
11	220	195	0.3	90	"
12	225	195	0.3	125	"
13	240	200	0.3	150	"
14	250	205	0.3		"
15	260	220	0.3		"
16	260	220	0.1		Oil

$C_{20}H_{16}$  Hydrocarbon—Fraction 6 was dissolved in ether and the solution was filtered from a slight amount of selenium. The concentrated filtrate crystallized at  $-20^{\circ}$ . After recrystallization from ether, rosettes of heavy, pointed crystals were obtained which melted at 125–127°.

$C_{20}H_{16}$ . Calculated. C 93.70, H 6.30, mol. wt. 256.13  
 Found. " 93.56, " 6.07, " " 257.7  
 " " 93.75, " 6.21

The molecular weight was determined in camphor.

This substance would not form a picrate of stable composition. The hydrocarbon itself crystallized from equimolecular proportions of picric acid and the substance dissolved in any one of a number of solvents which were tried.

*C<sub>21</sub>H<sub>24</sub> Hydrocarbon*—Fraction 5 did not give satisfactory material when the attempt was made to crystallize it directly from any solvent. It was therefore chromatographed in isohexane solution with Brockmann's alumina. The substance which passed through the column with the original solvent weighed 30 mg. and crystallized rather sharply from ether at  $-20^{\circ}$  in well shaped thin leaves. There was not sufficient material to permit carrying the purification further. It melted at  $70-81^{\circ}$ .

<i>C<sub>21</sub>H<sub>24</sub></i> .	Calculated.	C 91.24,	H 8.76,	mol. wt. 276.19
	Found.	" 91.07,	" 8.56,	" " 269.1

The molecular weight was determined in camphor.

The absorption spectrum of this substance showed a resemblance to that of fluorene but was displaced somewhat. The next 60 mg. of material coming from the chromatograph gave a crystalline hydrocarbon which corresponded in properties with the *C<sub>20</sub>H<sub>16</sub>* hydrocarbon reported above from Fraction 6.

*C<sub>24</sub>H<sub>30</sub> Hydrocarbon*—Fraction 7 could not be crystallized directly from any solvent in a satisfactory form. It was therefore chromatographed as in the case of Fraction 5. The first 30 mg. of material which emerged from the column yielded closely packed crystals which melted at  $91-98^{\circ}$ . After recrystallization from ether, rosettes of feathery curved needles were obtained which melted at  $100-101^{\circ}$ .

<i>C<sub>24</sub>H<sub>30</sub></i> .	Calculated.	C 90.50,	H 9.50,	mol. wt. 318.24
	Found.	" 90.71,	" 9.32,	" " 318.4

The molecular weight was determined in camphor.

The material coming from the succeeding chromatograph fraction crystallized sharply and appeared to be identical with the *C<sub>20</sub>H<sub>16</sub>* hydrocarbon from Fraction 6.

*C<sub>22</sub>H<sub>20</sub> Hydrocarbon*—Fraction 12 as directly obtained melted from  $105-135^{\circ}$ . After several recrystallizations from ether broad leaves were obtained which melted at  $154-155^{\circ}$ . Fraction 13 gave the same substance.

$C_{22}H_{20}$ .	Calculated.	C 92.91, H 7.09, mol. wt. 284.18
	Found.	" 93.19, " 6.95, " " 278.4
	"	" 92.88, " 7.04, " " 274.8

The molecular weight was determined in camphor.

This substance did not yield a picrate of constant composition but readily dissociated into the hydrocarbon and picric acid.

In the case of Fractions 10 and 11, it was difficult to obtain homogeneous material. The presence, however, of a hydrocarbon  $C_{21}H_{18}$  was suggested. The melting point of this material was not sharp but was in the region of 145–150°.

$C_{21}H_{18}$ .	Calculated.	C 93.28, H 6.72, mol. wt. 270
	Found.	" 93.16, " 7.10, " " 277.1
	"	" 92.96, " 6.75, " " 262.3

The molecular weight was determined in camphor.

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## THE ACONITE ALKALOIDS

### VII. ON STAPHISINE, A NEW ALKALOID FROM DELPHINIUM STAPHISAGRIA\*

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The alkaloids of the *Aconitum* species have been found to fall into two categories, the highly toxic aconitines and the much less toxic, simpler alkalamines such as atisine (1, 2), napelline (3), lucidusculine (4), and the more recently discovered kobusine (5). Although nothing has been accomplished as yet to establish any relationship between the two groups of alkaloids, their common occurrence in the related species of plants is suggestive of such relationship. Since the simpler alkaloids with fewer oxygen atoms should lend themselves more readily to degradative study, we have investigated the possibility of the occurrence of other alkaloids especially of this simpler group in the mother liquors which had accumulated in our isolation of delphinine from the seeds of *Delphinium staphisagria* (of commercial origin). In the older literature mention has been made of the occurrence of another crystalline alkaloid, delphisine (1, 2), a possible isomer of delphinine. Such an alkaloid has not been encountered in our work but the delphinine mother liquors were found to contain a relatively large amorphous fraction of alkaloids.

Following preliminary attempts by direct crystallization from a number of solvents, the use of the chromatographic procedure was found to yield the sharpest results. When a solution of the amor-

\* Our previous three papers on delphinine were presented under the heading, "Delphinine. I to III." Because of the expanding character of the general problem, all of these in future will be given under the heading, "The aconite alkaloids," Papers I to III of which have already appeared. The present paper, therefore, is Paper VII in the series.

phous alkaloids in benzene was passed through activated alumina, it was found that the first material to emerge could be crystallized readily from acetone. The yield of crystalline material in the first chromatograph fractions amounted to about 14 per cent of the amorphous fraction employed. Analysis of the different fractions indicated that we were dealing principally with a single alkaloid which crystallized from acetone as stout needles which did not possess a sharp melting point (200–208°). The rotation in 1 per cent benzene solution varied with different fractions or preparations at the most between  $[\alpha]_D = -152^\circ$  and  $-162^\circ$  and practically within the limit of observational error. The trivial name *staphisine* has been adopted.

The analysis of many samples of this substance fluctuated but little between the limits of 82.13 and 82.85 per cent for carbon, 9.47 and 9.77 for hydrogen, and 4.35 and 4.6 for nitrogen. No certain formulation was suggested by these figures. The molecular weight determinations in camphor varied from 538 to 572. The active hydrogen determination showed a practically negative result. The alkaloid contained oxygen and appeared to be an ether, since it did not react with hydroxylamine. It formed crystalline salts. Of these the *nitrate*, *hydrochloride*, and *hydrobromide* were readily obtained. The analyses of these salts while showing some variation appeared to suggest a formulation based on that of  $C_{22}H_{31}ON$  for the alkaloid itself. The possibility persisted that the so called staphisine could still be a persistent mixture of bases which are very difficult to separate. As a check on such a possibility, a special attempt was made to fractionate the alkaloid through its salts. This was accomplished first by conversion into the nitrate. The base which was obtained from the nitrate was fractionally crystallized. The first crystalline portion (C 82.62, H 9.73) was converted into the hydrochloride which was allowed to crystallize in fractions. The bases liberated from the successive hydrochloride fractions gave the following analyses: C 82.85, H 9.77 and C 82.38, H 9.54. No significant change in rotation was observed; *viz.*,  $[\alpha]_D = -158^\circ$  and  $-162^\circ$ .

In view of the persistent discrepancy in the analytical results obtained with the alkaloid itself and with its salts which were in approximate agreement among themselves, it appears possible that the parent alkaloid may possess the formula  $C_{22}H_{31}ON$  but

readily undergoes partial condensation between 2 molecules to form an ether or anhydro compound,  $C_{44}H_{60}ON_2$ , which forms an equilibrium mixture with the simpler alkaloid. In the case of the salts this equilibrium may be shifted in favor of the simpler hydroxy alkaloid itself. Though we do not suggest a direct analogy, the experience with this alkaloid recalls to mind such a bimolecular alkaloid derivative as pseudomorphine. Further study of our alkaloid will be required to establish the exact nature of the apparent mixture.

The alkaloid is tertiary and contains an N-methyl group and no methoxyl group. From its behavior on hydrogenation, it is unsaturated, but it proved to be difficult to measure the degree of unsaturation accurately.

The absorption approximated the requirements of one double bond on the  $C_{22}$  basis. Only a relatively small yield of a crystalline hydrogenation product could be isolated. As in the case of the staphisine used, the molecular weight determination was in agreement with a bimolecular formula. The analytical results supported such a formulation; viz.,  $C_{44}H_{64}ON_2$ . However, the general character of the ultraviolet absorption spectrum curve<sup>1</sup> obtained (Fig. 1) is definitely suggestive of the presence of two conjugated double bonds in the molecule. The position of the peak at 2670 Å. is more suggestive of two double bonds contained in one ring than in adjoining rings. Whether these conjugated double bonds occur in the so called parent alkaloid,  $C_{22}H_{31}ON$ , as such and therefore twice in the bimolecular alkaloid or only once in the latter cannot be decided as yet. With methyl iodide the reaction appeared to proceed in stages. The study of this reaction has not been entirely satisfactory because of analytical discrepancies which have been difficult to interpret. It appears, however, that the methylation slows up at an intermediate stage with formation of a monomethiodide of the ether,  $C_{44}H_{60}ON_2$ . This on longer heating with the reagent is completely methylated. Here again the analytical data showed some divergence from any consistent theoretical basis. Whereas the figures for the monomethiodide approached somewhat those required by the formula  $C_{44}H_{60}ON_2 \cdot CH_3I$  (or  $C_{42}H_{56}ON_2 \cdot CH_3I$ ?), those of the completely methylated

<sup>1</sup> The absorption spectra curves were very kindly determined by Dr. George I. Lavin of the Rockefeller Institute.

base approached the figures of the methiodide of the simpler hydroxy alkaloid,  $C_{22}H_{31}ON$  (or  $C_{21}H_{29}ON$ ?) with the formulation  $C_{22}H_{31}ON \cdot CH_3I$  (or  $C_{21}H_{29}ON \cdot CH_3I$ ).

In spite of the difficulty in deciding the question of the homogeneity and formulation of the alkaloid itself, it was apparent that it must belong in the same general category of alkaloids which includes atisine, napelline, etc. Since it can contain at most only

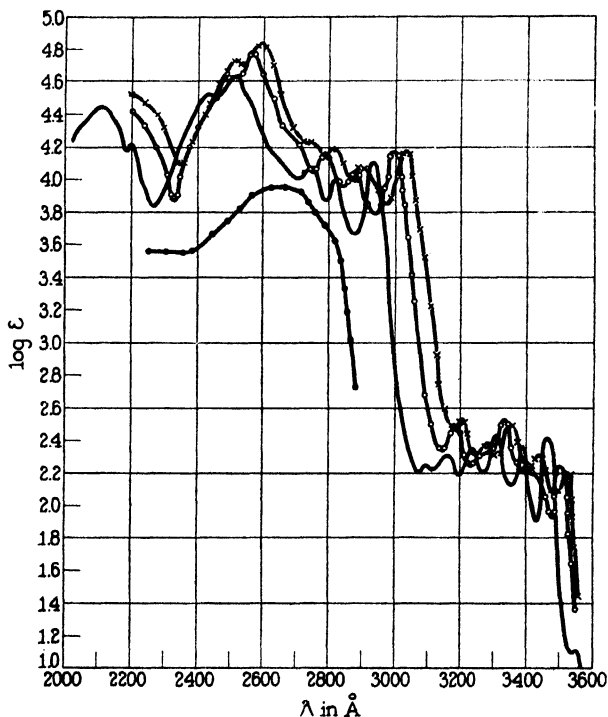


FIG. 1. Absorption spectrum curves. ● = staphisine, ○ =  $C_{16}H_{14}$  hydrocarbon, × =  $C_{19}H_{20}$  hydrocarbon, the solid line = phenanthrene.

1 oxygen atom and less than all of the other known alkaloids of this group which contain 2 or more oxygen atoms, it gave promise of being especially suitable for degradation experiments.

Aconitine and delphinine which contain so many hydroxyl groups have proved to be exceedingly difficult objects for degradative study. This has been especially true in all attempts to dehydrogenate them with selenium. On the other hand, in the case

of atisine, dehydrogenation has been reported by Lawson and Topps (1) to have yielded in addition to several nitrogen-containing substances a hydrocarbon mixture from which a hydrocarbon  $C_{17}H_{16}$  was isolated, possibly a substituted phenanthrene. Freudenberg and Rogers (3) have reported also the isolation of a hydrocarbon  $C_{17}H_{16}$  from napelline which they believed to be identical with that obtained from atisine.

We have made a preliminary study of the dehydrogenation of staphisine. Even if its homogeneity may be in question, the mixture could consist only of closely related alkaloids all built on the same ring system. The products of the reaction consisted of a basic and a neutral fraction. Investigation of the basic fraction has been for the time deferred. The neutral fraction consisted principally of a mixture of hydrocarbons. After preliminary chromatographic purification individual substances were separated by careful fractional distillation and a series of hydrocarbons was obtained. The first of these was isolated from a lower fraction first as the picrate which melted at  $129-131^{\circ}$ . The hydrocarbon regenerated from this melted at  $78-81^{\circ}$ . Analysis indicated a formulation  $C_{16}H_{14}$ . The ultraviolet absorption spectrum was characteristic of a phenanthrene. A comparison of the curve of this substance with that of phenanthrene<sup>2</sup> itself is given in Fig. 1 along with the curve of the  $C_{19}H_{20}$  hydrocarbon to be discussed below. Later fractions yielded a second hydrocarbon which was also purified as the picrate which melted at  $153-155^{\circ}$ . The hydrocarbon obtained from this did not melt sharply ( $55-63^{\circ}$ ). The analyses obtained from it and its picrate agreed with those of a tetramethylphenanthrene and its derivative.

A hydrocarbon was contained in larger amount in still later fractions which gave a picrate melting at  $142-144^{\circ}$ . The regenerated hydrocarbon melted at  $53-57^{\circ}$ . Analyses of the picrate and of the hydrocarbon approximated the figures required for either a tetramethyl- or pentamethylphenanthrene,  $C_{18}H_{18}$  or  $C_{19}H_{20}$ . Although the absorption curves of this and the preceding hydrocarbon have not been included in Fig. 1, visual comparison of the photographic plates taken of their continuous spectra obtained with a hydrogen discharge tube as light source with those of

<sup>2</sup> The curve of phenanthrene was replotted from the data of Mayneord and Roe (6).



phenanthrene and the hydrocarbon  $C_{19}H_{20}$  discussed below left no doubt that they must all be phenanthrene derivatives. An appreciable fraction followed the above hydrocarbon which yielded a crystalline hydrocarbon. The latter, contrary to the previous hydrocarbon, crystallized directly from ether. It melted at  $73-75^{\circ}$  and gave a picrate melting at  $143-144^{\circ}$ . Analysis fitted definitely with the figures for a pentamethylphenanthrene. The absorption spectrum curve of this hydrocarbon is presented in Fig. 1.

Among the last fractions a possible hexamethylphenanthrene,  $C_{20}H_{22}$ , was obtained. Its picrate formed orange needles which melted at  $135-137^{\circ}$ . The hydrocarbon liberated from this was only partly crystalline, so that its homogeneity was in question. Finally, a picrate was obtained from a final fraction, analysis of which agreed with the figures of a heptamethylphenanthrene but the amount available was too small for careful study.

From the above observations it appears to be definite that the hydrocarbon dehydrogenation products of staphisine are polymethylphenanthrenes, the individual identities of which remain to be determined. This result is in harmony with the conclusion that the probably related alkaloids atisine and napelline yield a hydrocarbon  $C_{17}H_{16}$  which has been regarded as a polymethylphenanthrene. However, as far as we have been able to find, no spectroscopic studies of the latter have been made to substantiate this conclusion. In the case of staphisine we have not obtained evidence of the presence of a  $C_{17}H_{16}$  hydrocarbon among its dehydrogenation products. In addition, there has been no suggestion of the production of any hydrocarbons analogous to the polycyclic fluorene hydrocarbons obtained from the veratrine alkaloids cevine and jervine and which give a Vanscheidt color test. It appears, therefore, that the phenanthrene ring system may be an essential portion of the make-up of staphisine and related alkaloids which in turn may be found to include the highly toxic alkaloids of the aconitine and delphinine group.

Finally, it should be recorded that a second very sparingly soluble alkaloid was encountered in very small yield from several fractions which came much later than staphisine in the chromatograph separation. This alkaloid was encountered first because of its very sparing solubility in acetone and ether. It melted with decomposition at  $300^{\circ}$ . No formulation has been derived

for it. Analysis showed C 80.69, H 8.84, N 4.43. It proved to be too sparingly soluble in camphor for a satisfactory molecular weight determination.

#### EXPERIMENTAL

15 gm. of the amorphous alkaloid mixture which resulted from the concentration and desiccation of the mother liquors from the preparation of delphinine were dissolved in 100 cc. of benzene and chromatographed through 500 gm. of activated  $\text{Al}_2\text{O}_3$  (Merck and Company, Inc., Rahway). Since elution of the column thirteen times with 100 cc. portions of benzene gave no residue, the eluent was changed to 1 per cent methyl alcohol in benzene. In this case also only very small amounts of substance totaling 0.3 gm. had begun to appear. Again the eluent was changed to 2 per cent methyl alcohol in benzene. The twenty-fourth fraction of 100 cc. of eluent withdrawn from the column yielded 0.24 gm. of residue which crystallized readily from the concentrated acetone solution. 0.16 gm. of crystals was collected which was recrystallized by concentration of the solution in hot acetone. It formed aggregates of prisms or needles which sintered above  $170^\circ$ , then gradually softened to a resin at about  $195^\circ$ , and melted at  $205\text{--}208^\circ$ .<sup>3</sup>

	$[\alpha]_D^{25} = -159^\circ$ ( $c = 1.09$ in benzene)
$\text{C}_{44}\text{H}_{40}\text{ON}_2$	Calculated. C 83.48, H 9.56, N 4.43, $\text{N}(\text{CH}_3)$ 4.75
$\text{C}_{22}\text{H}_{11}\text{ON}$	" " 81.17, " 9.61, " 4.31, " 4.61
	Found. " 82.36, " 9.47

The next fraction from the chromatograph yielded 0.41 gm. of residue which gave 0.29 gm. of crystals from acetone. The fraction which followed gave similarly 0.49 gm. of residue which crystallized readily from acetone. Yield, 0.4 gm. After recrystallization, it melted at about  $208^\circ$  after preliminary softening at from  $175\text{--}180^\circ$ .

Found. C 82.13, H 9.54, N 4.48

The succeeding fraction gave 0.4 gm. of crystals which after recrystallization melted at  $200\text{--}202^\circ$  after preliminary softening.

Found. C 82.19, H 9.61, N 4.51, 4.62

<sup>3</sup> All melting points given are micro melting points.

The molecular weight determined in camphor was 572.5. The Zerewitinoff determination showed but a trace of active H.

16.00 mg. substance: 0.07 cc.  $\text{CH}_4$  ( $24^\circ$ , 741 mm.); 0.27 cc. at  $90^\circ$ , 741 mm.  
Found at  $90^\circ$ , 0.068

The next fraction gave similar results. Finally, the twenty-ninth fraction gave 1.4 gm. of residue. From this, however, only 0.3 gm. of crystalline alkaloid was obtained from acetone. After recrystallization, it separated as needles which melted at  $198\text{--}202^\circ$  after preliminary sintering above  $188^\circ$ .

$[\alpha]_D^{25} = -152^\circ$  ( $c = 0.98$  in benzene)  
Found, C 82.17, H 9.52; C 82.32, H 9.58

This substance appeared identical in all respects with the material obtained from the very first crystalline fraction and showed no melting point depression.

The methoxyl and N-methyl determinations on several samples of alkaloid were as follows:

Found, (a)  $\text{OCH}_3$  1.26,  $\text{N}(\text{CH}_3)$  5.14; (b)  $\text{OCH}_3$  1.36,  $\text{N}(\text{CH}_3)$  5.14

In later experiments it was found possible to simplify the chromatographic separation by the use of much less  $\text{Al}_2\text{O}_3$ . When 50 gm. of the amorphous alkaloid fraction were dissolved in 100 cc. of benzene and passed through 600 gm. of alumina, it was not necessary to add methyl alcohol to the benzene. After 700 cc. of benzene had passed through the column, alkaloid began to emerge. A definite zone appeared with the subsequent 400 cc. of eluent which contained 8.65 gm. of alkaloid. This crystallized readily from acetone in a yield of 5.9 gm.

For later experiments 35 gm. of alkaloid obtained in this manner were recrystallized by solution in 100 cc. of benzene followed by the addition of an equal volume of acetone. 17.5 gm. of staphisine were obtained in this fraction.

$[\alpha]_D^{25} = -159^\circ$  ( $c = 1.16$  in toluene)  
Found, C 82.58, 82.73, H 9.71, 9.57, N 4.33, mol. wt. 538

A second fraction of 12.4 gm. was obtained from the mother liquor.

$[\alpha]_D^{25} = -156^\circ$  ( $c = 1.08$  in toluene)  
Found, C 82.29, H 9.62, N 4.37, mol. wt. 569

Staphisine did not appear to react with hydroxylamine, since it was recovered unchanged. When heated with methyl alcoholic HCl deep seated alteration occurred and no crystalline material could be recovered.

When heated with a 10 per cent methyl alcoholic KOH solution it remained mostly undissolved. After heating at 100° for 24 hours the collected material was recrystallized by solution in benzene, addition of methyl alcohol, and concentration. The substance had all the properties of staphisine.

C 82.61, H 9.74

*Attempt to Fractionate Staphisine*—5 gm. of staphisine  $[\alpha]_D^{25} = -159^\circ$  ( $c = 1.16$  in toluene) were dissolved in a solution of 7 cc. of acetic acid in 150 cc. of  $H_2O$ . 25 cc. of 10 per cent  $HNO_3$  were carefully added with shaking. The initial turbidity each time rapidly dissolved but was soon followed by copious separation of lustrous leaflets of the nitrate.

For analysis the salt was dried at 120° and *in vacuo*.

$C_{23}H_{31}ON \cdot HNO_3$ .	Calculated.	C 67.99, H 8.31, N 7.22
$C_{24}H_{30}ON_2 \cdot 2HNO_3$ .	"	" 69.61, " 8.24, " 7.39
	Found.	" 67.43, " 8.17, " 7.14

After recrystallization from hot water, it formed lustrous long platelets which softened to a resin at 236–243° with discoloration.

In the case of the second analysis which follows the salt was dried at 150° *in vacuo*.

C 67.84, H 8.21, N 7.02; C 68.10, H 8.22, N 7.12

The major portion of the nitrate was decomposed by continued shaking of the suspension in dilute NaOH with benzene until completely decomposed. The concentrated solution of the base in about 10 cc. of benzene was diluted with 20 cc. of acetone. The base crystallized readily as small needles which were collected with a benzene-acetone mixture. 2.1 gm. were contained in this fraction.

Found. C 82.62, H 9.73

2 gm. of the recovered alkaloid were suspended in methyl alcohol, and with chilling, HCl (1.19) was added drop by drop until solution was complete and the mixture was just acid to litmus.

The clear solution was concentrated *in vacuo* to remove the solvent. On addition of water to the syrupy residue crystallization of the salt began which soon formed a thick pap. This was redissolved by heating and in a volume of 15 to 20 cc. allowed to crystallize slowly. It finally became a mass of delicate needles. If chilled too quickly, it partly gelatinized. The yield of dry salt was 0.72 gm.

For analysis the salt was dried *in vacuo* at 120°. The dried salt was somewhat hygroscopic.

$C_{22}H_{31}ON \cdot HCl$ .	Calculated.	C 72.99, H 8.92, Cl 9.80
$C_{44}H_{60}ON_2 \cdot 2HCl$ .	"	" 74.85, " 8.86, " 10.05
	Found.	" 74.37, " 9.04, " 9.77
	"	" 74.17, " 8.87

This fraction was then recrystallized from water.

Found. C 73.84, H 9.08, Cl 9.96

The melting point varied with the rate of heating. When placed in the bath above 200°, it softened above 248° and effervesced at 256°.

The mother liquor from the first fraction of hydrochloride gradually deposited a second crop which increased by gradual cooling to 0°. After standing overnight the lustrous leaflets were collected by centrifugation. The yield was 0.45 gm. This fraction softened above 260° and effervesced at 265°.

Found. C 73.70, H 8.94, Cl 10.11

The alkaloid was recovered from each of these fractions of hydrochloride by decomposition in aqueous solution with alkali and extraction with benzene. The greatly concentrated solution was diluted with acetone. In each case copious crystallization of the regenerated alkaloid occurred.

The alkaloid from the first hydrochloride fraction showed  $[\alpha]_D^{25} = -158^\circ$  ( $c = 1.11$  in toluene) and gave the following analysis.

Found. C 82.85, H 9.77

The alkaloid from the second hydrochloride showed  $[\alpha]_D^{25} = -162^\circ$  ( $c = 0.98$  in toluene) and gave the following analysis.

Found. C 82.38, H 9.54

The alkaloid which was recovered from the mother liquor of the second hydrochloride fraction gave similar figures.

Found. C 82.31, H 9.72

It is thus apparent that no significant separation of any possible individual alkaloids was accomplished by this procedure.

The hydrobromide of staphisine was also prepared. This salt separated by the addition of a drop of water to its concentrated solution in acetone followed by ether. It crystallized with water of crystallization as minute leaflets which softened to a resin at 255–258°.

For analysis it was dried at 120° *in vacuo*. The result was not altered by drying at 150°.

$C_{22}H_{31}ON \cdot HBr$ .	Calculated.	C 65.00, H 7.94, Br 19.68
$C_{44}H_{60}ON_2 \cdot 2HBr$ .	"	" 66.47, " 7.87, " 20.12
	Found.	" 64.95, " 7.92, " 20.45
	"	" 64.97, " 7.84

*Hydrogenation of Staphisine*—0.2 gm. of the alkaloid was hydrogenated under 3 atmospheres pressure with 50 mg. of platinum oxide catalyst (Adams and Shriner) in methyl alcohol to which a drop of concentrated HCl had been added. Without HCl, hydrogenation did not appear to go beyond the reduction of the catalyst. After reduction of the catalyst the absorption was gradual but practically completed within 2 hours. However, the operation was continued for 24 hours. The absorption beyond the catalyst requirements was about 12.5 cc. or not quite 1 mole for the formula  $C_{22}H_{31}ON$  or 2 moles for the double formula  $C_{44}H_{60}ON_2$ . After recovery of the base from the reaction mixture it formed a resin which slowly crystallized from acetone. 47 mg. were obtained. After recrystallization by solution in benzene and concentration followed by addition of acetone to remove the benzene, it gradually crystallized as minute clusters of micro crystals followed by large broad needles possibly of an isomeric substance.

The following analyses are for different preparations. Although they were in fair agreement, the melting points of the individual preparations varied from 205–209° to 252–254°. Either a varying mixture of isomers was responsible for this or the substance is polymorphic. It did not contain solvent of crystallization.

$C_{44}H_{64}ON_2$ .	Calculated.	C 82.95, H 10.13, mol. wt. 636.53
$C_{22}H_{33}ON$ .	"	" 80.67, " 10.16, " " 327.26
	Found. (a)	" 82.50, " 10.08, " " 664
	" (b)	" 82.19, " 10.08
	" (c)	" 81.52, " 10.15, mol. wt. 642
	" (d)	" 82.44, " 9.78

Although like the parent base it reacted with methyl iodide, the resulting product could not be crystallized.

*Staphisine and Methyl Iodide*—The reaction between the alkaloid and methyl iodide appeared to go in two stages but the interpretation of the reaction is not certain.

0.33 gm. of staphisine was sealed with several cc. of methyl iodide. On reaching room temperature, the chilled gelatinous mass formed a clear solution but rapidly began to deposit crystals. The mixture was heated at 100° for 2 hours. The contents of the tube consisting of a pap of leaflets and needles were collected with benzene. 0.17 gm. was obtained. This fraction was recrystallized from a methyl alcohol-ether mixture. The substance consisted chiefly of aggregates of needles with a few scattered minute prisms and effervesced at 250° after gradual softening above 240°.

$C_{44}H_{80}ON_2 \cdot 2CH_3I$	Calculated.	C 60.24, H 7.26, I 27.70, N(CH <sub>3</sub> ) 6.56
$C_{32}H_{51}ON \cdot CH_3I$	"	" 59.08, " 7.33, " 27.17, " 6.43
$C_{31}H_{49}ON \cdot CH_3I$	"	" 58.25, " 7.12, " 28.01, " 6.63
	Found.	" 58.98, " 7.41, " 28.25, " 6.22

In the procedure for OCH<sub>3</sub> determination, only 1.13 per cent was found which apparently was due to the N(CH<sub>3</sub>) group.

The mother liquor from the above reaction product gave a resin on concentration which crystallized from acetone. This proved to be much less soluble in methyl alcohol than the above substance. On recrystallization from this solvent, it formed minute four-sided platelets or prisms.

From the analysis it was not possible to decide whether the substance was the monomethiodide of the bimolecular base,  $C_{44}H_{80}ON_2$  (or  $C_{42}H_{56}ON_2$ ) contaminated with other substances or a homogeneous substance with another formulation.

$C_{44}H_{80}ON_2 \cdot CH_3I$	Calculated.	C 69.73, H 8.20, I 16.39, N(CH <sub>3</sub> ) 5.82
$C_{42}H_{56}ON_2 \cdot CH_3I$	"	" 69.13, " 7.97, " 17.00, " 6.04
	Found.	" 68.55, " 8.17, " 6.37

The OCH<sub>3</sub> found was negligible; *viz.*, 0.88.

After two recrystallizations from methyl alcohol, no appreciable shift could be detected. It effervesced at 255° after softening above 245°.

Found. C 68.60, H 8.34, I 17.20

A 0.1 gm. sample of the second reaction product was heated longer with methyl iodide for 16 hours at 100°. At first it almost all dissolved but this was gradually followed by a deposit of crystals. The collected material (0.12 gm.) was recrystallized from a methyl alcohol-ether mixture. 88 mg. were obtained which effervesced at 250°.

Found. C 58.40, H 7.29, I 27.96

When the reaction was continued at 100° for 60 hours, a similar result was obtained, although accompanied by some decomposition.

When staphisine and methyl iodide were allowed to stand at room temperature for 24 hours, lustrous, four-sided platelets separated which softened gradually to a resin from 240–250°.

The substance was dried *in vacuo* at 100°.

Found. C 69.37, H 8.22, I 16.35

In another experiment the reaction product between staphisine and methyl iodide at 100° was dissolved in hot water containing dilute acetic acid. On cooling, copious crystallization of minute needles occurred; these were collected with water.

For analysis the substance was dried *in vacuo* at 110°.

Found. C 58.78, H 6.83, I 27.29, 27.56

*Dehydrogenation of Staphisine*—20 gm. of staphisine were well mixed with 60 gm. of selenium and the mixture was heated in an apparatus such as that previously employed and through which a steady stream of nitrogen was passed. The temperature was raised to 340° in approximately half an hour and held there for 2 hours.

The volatile material which condensed in the ice trap has not thus far yielded to study but this will be continued. The residue from the dehydrogenation was finely pulverized and well extracted with ether. Upon evaporation of the ether a residue of 13.5 gm. was obtained. It was dissolved in 100 cc. of ether and 50 cc. of 10 per cent HCl were added. After shaking, the neutral ether extract was separated from the resinous aqueous mixture and set aside to be treated as described below. The resinous solid material in the aqueous layer was extracted with chloroform. Nothing further could be obtained from the acid aqueous layer.



The chloroform solution was shaken with dilute NaOH and then evaporated to dryness. The residue contained 6.5 gm. of basic material which has not been investigated thus far.

The above neutral ether extract was dried over  $K_2CO_3$  and evaporated to dryness. Benzene was then added and the evapora-

TABLE I  
*Fractionation of Hydrocarbon Mixture.*

Fraction No.	Bath temperature	Column temperature	Weight of fraction	Physical character	Analysis	
					C	H
	°C.	°C.	mg.		per cent	per cent
1	180	130	60	Oil	89.37	9.47
2	183	132	100	"	89.99	10.03
3	183	140	100	Crystals	90.99	8.83
4	183	147	170	"	92.10	7.92
5	185	153	160	"	92.09	7.88
6	185	160	130	"	92.09	7.92
7	185	160	130	Oil		
8	190	166	130	"		
9	191	171	130	Crystals	92.17	7.78
10	191	172	130	Oil		
11	191	172	130	Crystals	91.84	8.06
12	191	172	130	"		
13	191	172	130	"	91.80	8.17
14	191	172	130	"		
15	191	172	130	"	91.99	8.07
16	191	172	130	"		
17	191	172	130	"	91.93	7.98
18	191	172	110	Oil		
19	193	167	130	Crystals	91.82	8.28
20	193	167	130	"		
21	195	167	130	Oil	90.79	8.07
22	200	170	130	"	90.78	8.30
23	220	205	130	Crystals containing Se		

tion repeated. The residue was then dissolved in 75 cc. of benzene and chromatographed with 500 gm. of activated alumina in benzene. As soon as material began to emerge from the column, 175 cc. were collected. This yielded 3.83 gm. of oily residue which was placed in a sublimation apparatus and distilled up to a bath temperature of 200° under 0.2 mm. pressure. The distillate

weighed 3.3 gm. This was fractionated in a microfractionating apparatus with a column 22 cm. in length and under 0.2 mm. pressure. The fractionation data are given in Table I.

Fractions 1 and 2 appeared from the analysis to consist mostly of incompletely dehydrogenated material and could not be crystallized. Fractions 3 and 4 both yielded a crystalline identical picrate.

*Dimethylphenanthrene, C<sub>16</sub>H<sub>14</sub>*—Fraction 4 was treated with 100 mg. of picric acid in benzene and the mixture was chilled to 8°. 130 mg. of yellow needles were obtained which melted at 122–126°. After recrystallization from acetone at –20° the material melted at 129–131°.

C <sub>16</sub> H <sub>14</sub> ·C <sub>6</sub> H <sub>3</sub> O <sub>7</sub> N <sub>3</sub> .	Calculated.	C 60.66, H 3.93
	Found.	" 60.66, " 3.93

The picrate was decomposed with ether and 2 per cent NaOH solution. The hydrocarbon residue obtained from the ether extract was distilled under reduced pressure. The crystalline distillate melted at 78–81° with previous sintering at 73°. It showed the characteristic absorption spectrum of a phenanthrene hydrocarbon (Fig. 1).

C <sub>16</sub> H <sub>14</sub> .	Calculated.	C 93.15, H 6.85, mol. wt. 206.11
	Found.	" 92.90, " 6.94, " " 203.6

The molecular weight was determined in camphor by the Rast method.

*The Hydrocarbon C<sub>18</sub>H<sub>18</sub>*—Both Fractions 5 and 6 gave fair analytical data without further purification for a hydrocarbon, C<sub>18</sub>H<sub>18</sub>. Upon further examination both proved apparently to be mixtures of isomeric substances but yielded the same picrate. Fraction 6 was treated with 100 mg. of picric acid and the mixture crystallized from acetone at –20°. 95 mg. of picrate were obtained which melted at 133–144°. After two recrystallizations from acetone, the light orange needles melted at 153–155°.

C <sub>18</sub> H <sub>18</sub> ·C <sub>6</sub> H <sub>3</sub> O <sub>7</sub> N <sub>3</sub> .	Calculated.	C 62.18, H 4.56
	Found.	" 62.10, " 4.52
	"	" 61.95, " 4.13

The hydrocarbon recovered from the picrate was an oil. After distillation the material, although still an oil, could be crystallized

from isopentane. Since it melted at a low temperature, it was redistilled under reduced pressure in order to remove the solvent. The distillate as such melted at 55–63°. There was not sufficient material for further recrystallization.

$C_{18}H_{18}$ . Calculated, C 92.25, H 7.75; found, C 91.94, H 7.60

*The Hydrocarbon  $C_{18}H_{18}$  or  $C_{19}H_{20}(?)$* —Upon investigation Fractions 9 to 14 appeared to consist in largest part of the same hydrocarbon. The material directly from the still gave analytical data in somewhat better agreement with the  $C_{18}H_{18}$  formula and was undoubtedly a mixture of closely related substances.

Fraction 9 with 100 mg. of picric acid was crystallized from acetone at  $-20^{\circ}$ . 140 mg. of orange needles were obtained which melted at 136–139°. After two recrystallizations from acetone at  $0^{\circ}$  the picrate melted at 142–144°.

$C_{18}H_{18} \cdot C_6H_3O_7N_3$ .	Calculated.	C 62.18,	H 4.56
$C_{19}H_{20} \cdot C_6H_3O_7N_3$ .	"	" 62.87,	" 4.86
	Found.	" 62.64,	" 4.72

The recovered hydrocarbon after distillation melted at 40–46°. It could not be crystallized from ether but formed diamond-shaped leaflets from isopentane which melted at 45–48°. When redistilled under reduced pressure in order to remove the solvent, the crystalline distillate melted at 53–57°.

$C_{18}H_{18}$ .	Calculated.	C 92.25,	H 7.75,	mol. wt.,	234.14
$C_{19}H_{20}$ .	"	" 91.88,	" 8.12,	" "	248.16
	Found.	" 92.26,	" 7.93,	" "	224.8

The molecular weight was determined in camphor.

Both the hydrocarbon and its picrate gave a depression with the  $C_{18}H_{18}$  hydrocarbon and its picrate from Fraction 6.

Fraction 11 yielded a picrate which melted at 142–144° and gave no depression with the above picrate from Fraction 9.

Found. C 62.87, H 4.84

The hydrocarbon obtained from this picrate crystallized in diamond-shaped leaflets from isopentane and melted at 48–51° and after distillation at 52–56°. It gave no depression with the hydrocarbon from Fraction 9.

Found. C 92.05, H 8.13, mol. wt. 223.5

The picrate from Fraction 14 melted at 138–139°.

Found. C 62.87, H 4.47

The hydrocarbon after distillation melted at 48–53°.

Found. C 91.94, H 7.99

*The Hydrocarbon C<sub>19</sub>H<sub>20</sub>*—The fractions from No. 15 on crystallized directly from ether as contrasted with those below Fraction 15 which crystallized only from isopentane. Further purification did not change the analytical data from those obtained in the original fractions in any of the fractions up to Fraction 21. They appeared therefore to be mixtures of isomeric compounds.

Fraction 15 was recrystallized from ether at –20°. Broad, thin diamond-shaped plates were obtained which melted at 68–71°. A further recrystallization gave material which melted at 70–72°.

C <sub>19</sub> H <sub>20</sub> .	Calculated.	C 91.88, H 8.12, mol. wt. 248.16
	Found.	" 92.13, " 7.90, " " 244.7

The picrate crystallized from acetone as orange needles which melted at 143–144°.

C <sub>19</sub> H <sub>20</sub> ·C <sub>6</sub> H <sub>3</sub> O <sub>7</sub> N <sub>3</sub> .	Calculated.	C 62.87, H 4.86
	Found.	" 62.81, " 4.60
		" 62.51, " 4.64

Fraction 19 yielded a picrate from acetone which melted at 141–142°.

Found. C 62.99, H 4.84

The hydrocarbon from this picrate after distillation melted at 55–63°.

Found. C 91.89, H 8.00, mol. wt. 238.7

This hydrocarbon after two recrystallizations from ether melted at 73–75° and gave no depression with the hydrocarbon from Fraction 15.

Fraction 21 gave a picrate which melted at 138–140°.

Found. C 63.00, H 4.87

*The Hydrocarbon C<sub>20</sub>H<sub>22</sub> (?)*—A definite shift in results appeared with Fraction 22. When this was treated with 100 mg. of picric

acid in benzene, it crystallized at 0°. After two recrystallizations from acetone, 48 mg. of orange needles which melted at 135–137° were obtained.

$C_{20}H_{22} \cdot C_6H_5O_7N_3$ .	Calculated.	C 63.51, H 5.13
	Found.	" 63.37, " 4.84
	"	" 63.79, " 4.45

The hydrocarbon regenerated from the picrate even after distillation was only partially crystalline and obviously not homogeneous.

$C_{20}H_{22}$ .	Calculated.	C 91.54, H 8.46
	Found.	" 91.92, " 8.30
	"	" 92.08, " 8.29

Fraction 23 contained selenium. It was dissolved in ether and filtered. The picrate obtained from this was crystallized from ether. After two recrystallizations from alcohol, orange-brown flat blades were obtained which melted at 138–142°. The analytical figures agreed with those required for the picrate of a  $C_{21}H_{22}$  hydrocarbon but there was not sufficient material for extended investigation.

$C_{21}H_{22} \cdot C_6H_5O_7N_3$ .	Calculated.	C 64.38, H 5.01
	Found.	" 64.39, " 5.22

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## THE EFFECT OF CERTAIN REAGENTS ON THE ACTIVITY OF BIOTIN

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In the present communication we are reporting the action of various reagents on the activity of biotin. The criterion used was the effect upon yeast growth activity (1). Preliminary information was thus obtainable with a minimum amount of material. The results of such inactivation experiments must, of course, be interpreted cautiously. When inactivation has been brought about, it is justifiable to assume that a change in the molecule has undoubtedly occurred, but when no inactivation has taken place, the possible formation of an active derivative or degradation product cannot be excluded. The data which we have obtained in these inactivation experiments are summarized in Table I. Reference to the work of other investigators who have tested the effect of some of these reagents on the compound, and assayed either for coenzyme R activity (*Rhizobium* growth), vitamin H activity (egg white injury curative action), or for biotin activity (yeast growth), is also given in Table I.

Prior to the isolation of the crystalline material (9), many experiments were carried out in this laboratory on fractions (containing from 1 to 20  $\gamma$  of biotin per mg.) prepared from a liver concentrate. All of these experiments have been repeated on the crystalline biotin. The majority of the experiments done on the crystalline material were carried out on 1 or 2 cc. aliquots of a solution containing 12.5  $\gamma$  of biotin (9) per cc. The standard solution of biotin was evaporated to dryness in a test-tube and the desired reagent added. Upon completion of the reaction, the solution was concentrated at reduced pressure if necessary. It was then neutralized and made up to a volume of 5 cc., 1 cc. of

TABLE I  
Summary of Inactivation Experiments

Reagents used	Inactivation observed		Bibliographic reference No.	Reagents used	Inactivation observed		Bibliographic reference No.
	Present study	Other workers			Present study	Other workers	
Air or O <sub>2</sub>	—	—	2	(CH <sub>3</sub> CO) <sub>2</sub> O-NaOH	—	±†	7
5% H <sub>2</sub> O <sub>2</sub>	+	+	3	(CH <sub>3</sub> CO) <sub>2</sub> O-CH <sub>3</sub> COOH	—		
Ether peroxides	+			with Zn	—		
H <sub>2</sub> -Pd	—			CH <sub>2</sub> =C=O	—	+†	8
H <sub>2</sub> -PtO <sub>2</sub>	—	*		C <sub>6</sub> H <sub>5</sub> COCl-NaOH	—	§	
Na in liquid NH <sub>3</sub>	—			C <sub>6</sub> H <sub>5</sub> COCl-C <sub>6</sub> H <sub>5</sub> N	—	±§	5
Chloramine-T	+			C <sub>6</sub> H <sub>5</sub> NCO	—		
Aqueous Br <sub>2</sub>	+			NaOH	+		
SO <sub>2</sub>	+	—†	3	KOH	+		
HCl in CH <sub>3</sub> OH	—	—	4, 5	KOCH <sub>3</sub>	+		
CH <sub>2</sub> N <sub>2</sub>	—	—	5	Methylation	—		
HCl	+			Benzoylation	—		
HCl-HCOOH	+			CH <sub>2</sub> O	+	+	8
H <sub>2</sub> SO <sub>4</sub> , concentrated	—			HONO	+	+	4, 8
CH <sub>3</sub> COOH-H <sub>2</sub> SO <sub>4</sub>	—			Ninhydrin	—		
CH <sub>3</sub> COCl-NaOH	—	±†	6	NH <sub>2</sub> OH	—		
				p-NO <sub>2</sub> C <sub>6</sub> H <sub>4</sub> NHNH <sub>2</sub>	—		
				Ultraviolet light irradiation	—		

\* This experiment was performed on 1.5 mg. of material by Dr. Klaus Hofmann with the use of a Warburg vessel as the micro hydrogenation apparatus.

† With coenzyme R (biotin) little inactivation with SO<sub>2</sub> was found.

‡ Despite conflicting reports we have been unable to inactivate biotin by acetylation.

§ It is reported (8) that vitamin H (biotin) is inactivated by benzoylation, but the conditions used were not specified. Also, biotin is reported (5) to be inactivated by benzoyl chloride in pyridine and this inactive product may be reactivated to the extent of 20 per cent by saponification with sodium ethylate.

|| It was found (4) that the rate of inactivation was approximately equal to that of the destruction of  $\alpha$ -amino acids.

which was used for assay. The possibility that the observed effect was caused by one reagent and not by the combination of

reagents was minimized by parallel control experiments in which the effect of each individual reagent was determined.

*Effect of Acid and Alkali*—It was noted that in many experiments involving vigorous treatment with acids or alkalis there was some loss of activity, usually attributable solely to the effect of these reagents. In a study of the effect of acid, it was found that the use of 20 per cent HCl over fairly long periods of time was required to inactivate the samples completely. The following results were obtained in experiments in which 25  $\gamma$  of biotin in 2 cc. of 20 per cent HCl at 120° were used.

Time	Per cent of activity destroyed
6 hrs.	50
12 "	65
24 "	90
48 "	100

A mixture of equal volumes of concentrated HCl and 90 per cent formic acid at 120° had the same effect as HCl alone.

The action of alkali results in greater inactivation than corresponding strengths of acid. For example, 40 to 60 per cent inactivation was brought about by the action of 1 *N* KOH at 120° for 17 hours. Similar inactivation was caused by refluxing the biotin with 5 per cent potassium methylate for 1.5 hours. Longer periods of treatment or stronger concentrations of alkali led to complete inactivation. Thus, in contrast to other data in the literature, biotin does not appear to be as stable to acid and to alkali as has been assumed.

*Effect of Oxidizing and Reducing Agents*—Prolonged aeration of either an acid or an alkaline solution of biotin with air or O<sub>2</sub> had no appreciable effect, but the use of stronger oxidizing agents quickly destroyed the activity. For instance, treatment with 5 per cent H<sub>2</sub>O<sub>2</sub> completely destroyed the activity as did the use of peroxide-containing ether. Aqueous solutions of Br<sub>2</sub> also completely destroyed the activity. This last may be attributable to the general oxidizing properties of the reagent as well as to its possible addition to an unsaturated linkage.

Biotin was not affected by treatment with hydrogen, even in the presence of Pd or PtO<sub>2</sub> catalysts, nor was its activity destroyed by reduction with Na in liquid NH<sub>3</sub>, or Na in alcohol.

*Question of Presence of an  $\alpha$ -Amino Acid Grouping*—The fact



that biotin has been found by others to be inactivated by nitrous acid has led to the assumption that biotin is very likely an  $\alpha$ -amino acid ((4) and Table I, foot-note ||). We have confirmed this inactivation with nitrous acid and also the inactivating effect of formaldehyde. With the latter reagent it was necessary to heat the biotin with a 40 per cent solution of formaldehyde to bring about extensive inactivation. In addition, we observed that biotin is rapidly and completely inactivated by chloramine-T, which would be expected if it were an  $\alpha$ -amino acid but which is also in accord with the ease of oxidation of biotin. We have found, however, that ninhydrin has no effect whatsoever on the activity of the compound, and the absence of any reaction with this reagent strongly indicates that biotin is not an  $\alpha$ -amino acid. Experiments have been carried out at pH 4.7 (recommended for the majority of the known amino acids) (10) and at pH 2.5 (recommended for hexone bases) as well as at pH 6.7.

*Acetylation and Benzoylation*—If an amino, imino, or primary or secondary alcohol grouping containing an active hydrogen was present, the formation of acetyl or benzoyl derivatives would be expected. We have attempted acetylation under a variety of conditions, including the use of acetic anhydride-sodium hydroxide, acetyl chloride-sodium hydroxide, acetic acid-sulfuric acid, and reductive acetylation in acetic acid-acetic anhydride-zinc. In no case was there an appreciable amount of inactivation, beyond that attributable to the effect of the alkali when it was used. We have likewise been unable to inactivate biotin by the use of ketene.

Benzoylations were also attempted by use of benzoyl chloride in NaOH and in KOH solutions. No inactivation resulted. Neither have we been able to effect any inactivation by the use of benzoyl chloride and pyridine.

In addition to these experiments, biotin was treated with phenyl isocyanate in alkaline solution as well as under anhydrous conditions with heating but no inactivation resulted.

*Methylation and Benzylation*—Methylation and benzylation experiments were carried out with the use of Na in alcohol, followed by the addition of methyl iodide or benzyl chloride. Similar experiments with K were also done. In all cases the effect of the treatments was equal only to that of the alkali alone.

*Effect of Carbonyl Reagents*—The action of *p*-nitrophenylhydra-

zine in acidified alcohol and of hydroxylamine in alkaline solution also failed to bring about inactivation, thus indicating the absence of an aldehyde or ketone.

The authors wish to express their appreciation to Miss Eleanor Hague for carrying out the numerous biotin assays.

#### SUMMARY

It has been shown that biotin can be inactivated by vigorous treatment with acid and alkali.

Although biotin is inactivated by many reagents known to react with  $\alpha$ -amino acids, its activity is not affected by ninhydrin. The latter fact strongly indicates that biotin is not an  $\alpha$ -amino acid. The activity of biotin is not destroyed by the use of acylating or alkylating reagents, nor by the use of carbonyl reagents.

It has been shown that biotin contains an easily oxidizable group or groups.

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# THE ISOLATION OF PURIFIED PROTEIN DERIVATIVES AND CARBOHYDRATES FROM TUBERCULINS AND THEIR BIOLOGICAL PROPERTIES

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The studies of Seibert and Munday (1) have demonstrated that the active principle of tuberculin is in the protein fraction. Recently, Seibert, Pedersen, and Tiselius (2) have shown that, when protein is isolated from tuberculin and fractionated into proteins of definite molecular structure by the aid of the Tiselius electrophoresis apparatus and studied in the Svedberg ultracentrifuge, one protein will elicit a stronger reaction than the other proteins when inoculated intracutaneously in a sensitized animal.

We thought it would be of interest to separate the proteins from the tuberculins produced by Ra (rough avirulent) and Rv (rough virulent) microorganisms obtained from the single parent strain H-37. Seibert (3) had isolated PPD by ultrafiltration from tuberculins produced by both virulent and avirulent microorganisms, but these were not obtained from a single parent strain.

Because we lacked an ultracentrifuge or a Tiselius electrodialysis apparatus, we were limited in our efforts to make a comparison of our PPD and carbohydrates after separating them by ultrafiltration or dialysis, but our results were, nevertheless, significant and are here reported.

*Preparation of Tuberculin*—10 liters of medium<sup>1</sup> were prepared and bottled in 8 ounce bottles, each containing 100 cc. 50 of these bottles were seeded with a 1 cm. loop of pellicle of Ra microorganisms which had been growing on a similar medium for 3

<sup>1</sup> The modified Proskauer and Beck medium consisted of monopotassium phosphate 5.0 gm., asparagine 5.0 gm., magnesium sulfate 0.6 gm., magnesium citrate 2.5 gm., glycerol 20.0 cc., and H<sub>2</sub>O to 1000.0 cc.

weeks. The other 50 bottles were similarly seeded with Rv microorganisms grown on the same medium for the same length of time.

All of the bottles were incubated at 37.5° for 8 weeks before the organisms of each variant were harvested separately, and the resulting pooled tuberculins made up to their original volumes of 5000 cc. The cultures of microorganisms differed grossly in physical appearance. The Ra variant was dry and brittle with a very dense and heaped-up growth, while the Rv growth was moist and tenacious, and grew very thinly and diaphanously on the sides of the bottles.

The yields of the microorganisms dried *in vacuo*, given in gm. per 5 liters, were as follows: Series I, Ra 18.99, Rv 16.61; Series II, Ra 19.54, Rv 17.12.

The PPD samples were removed according to the method of Seibert (3). 5 liters of each tuberculin which were previously heated to 55° for 1 hour were filtered through alundum crucibles coated with 13 per cent guncotton solution. (This differs from Seibert's procedure in that she heated the tuberculins plus organisms in an Arnold sterilizer for 3 hours.) Separate batteries of crucibles containing each tuberculin were attached to a manifold and connected to the same suction line, so that both tuberculins were filtered simultaneously and at approximately the same rate of flow, until there remained about 100 cc. of concentrated tuberculins of a dark brown color. These tuberculins were refiltered through Berkefeld filters of the N type to remove any surviving bacterial bodies and débris.

4 volumes of 50 per cent trichloroacetic acid were added to the filtrates which were then placed in an ice box overnight. In the morning the precipitated tuberculins were centrifuged at high speed for 20 minutes and the supernatant fluid decanted. Then, for seven successive times, the resulting sediments were partially dried *in vacuo* and washed with diethyl ether until neutral to litmus. Finally the sediments were dried *in vacuo* and weighed. The following yields of buff-colored PPD are given in gm. per 5 liters: Series I, Ra 0.45, Rv 1.00; Series II, Ra 0.61, Rv 1.30.

*Isolation of Carbohydrates*—After the removal of protein, the filtrate from each variant was neutralized individually with N NaOH. 4 volumes of 95 per cent alcohol were added to each,

producing flocculent precipitates. These flasks plus their precipitated contents were placed in an ice box overnight. In the morning, the alcohol was decanted and the remaining precipitates plus residual alcohol were centrifuged at high speed for 20 minutes. The supernatant alcohol was again decanted, and the resulting sediments washed with 95 per cent alcohol until free of alkali, and then washed with ether until completely dehydrated. Any remaining ether was removed *in vacuo*. The yields of carbohydrate, given in gm. per 5 liters, were as follows: Series I, Ra 0.20, Rv 0.37; Series II, Ra 0.22, Rv 0.45.

*Nitrogen Determinations on Various Fractions*—The per cent nitrogen, determined by the micro-Dumas procedure, was as follows: PPD, Rv 15.01, Ra 14.53; carbohydrate, Rv 0.18, Ra 0.08.

*Biological Tests with PPD*—Twenty male guinea pigs were inoculated subcutaneously with living suspensions of Ra and Rv microorganisms, ten guinea pigs with 2.5 mg. of the Ra variant, and ten with 2.5 mg. of the Rv variant. 3 weeks after inoculation the animals were tested with 0.1 cc. of 5 per cent OT and they all reacted with approximately  $12 \times 12$  mm. areas of erythema and induration, with small necrotic centers.

The PPD samples were dissolved in normal saline, so that each 0.1 cc. contained 92  $\gamma$  of PPD. Every animal in each series that had been inoculated with Ra and Rv variants was tested on one side with Ra PPD and on the other with Rv PPD. All animals demonstrated approximately the same reactions with both PPD solutions.

In the belief that we might be able to show specificity by using more dilute PPD solutions, the same series of animals were again tested with both strains of PPD, but this time with a concentration of 27.7  $\gamma$ . Again the skin tests were identical, as in the previous series of tests.

*Skin Test with Carbohydrates*—The same series of infected animals were tested with 27.7 and 92  $\gamma$  dilutions of both carbohydrates, but neither was capable of eliciting a skin reaction. However, with 0.1 mg. we were able to elicit a reaction that had a central area of necrosis but without the typical surrounding area of erythema and induration that accompanies a tuberculin reaction.

Two male goats of approximately the same size, age, and color were used for a skin test with human, bovine, and avian tuberculins

and did not react. From the jugular vein of each goat, 7 to 10 cc. of blood were removed aseptically; the blood was permitted to coagulate, and then it was rimmed and centrifuged. The sera were removed aseptically and stored in sterile ampules to be used as control sera throughout the experiment.

The goats were inoculated intravenously on 2 alternate days, one goat with Ra and the other with Rv living microorganisms suspended in normal saline, each animal receiving 5 cc., or approximately 10 mg., of its appropriate variant. 1 month after the last inoculation, each goat was bled and tested for precipitins and complement-fixing antibodies. The blood of both goats had demonstrable fixing antibodies when titrated against antigens prepared from Ra and Rv microorganisms; both also reacted with a definite degree of specificity.

Precipitins could be demonstrated only with the carbohydrate fractions and not with the PPD fractions. More will be said about the precipitin reaction later.

2 weeks later, or 6 weeks after the last inoculation with microorganisms, the two goats were bled to death from their jugular veins, and the sera separated aseptically from the clot to be stored in sterile ampules.

*Results of Precipitant Tests*—Table I demonstrates the strong reaction of Ra carbohydrate with its homologous serum, and conversely, the stronger reaction of Rv carbohydrate with serum homologous to the Rv carbohydrate. It will also be noted that the Ra serum reacts more strongly with both Ra and Rv carbohydrates than does the Rv serum.

On the strength of the above findings, we thought that the specificity might be caused by a predominance of one form of carbohydrate. To test this possibility, both sera were submitted to Dr. Heidelberger of Columbia University who tested them against various carbohydrates which he and Dr. Menzel had isolated from the tubercle bacilli Strain H-37 (4).

*Carbohydrates*—520-C is relatively highly dextrorotatory, high in pentose; 520-B2a, low dextrorotatory, high in pentose; 520-B2a", comprises both of the specificities of the two preceding fractions; 520-B2-C, inactive against rabbit and horse antisera, high in phosphorus; 520-V1-C, representative of the fractions highly soluble in organic solvents.

TABLE I  
*Results of Precipitant Tests*

All serum and carbohydrate samples measured 0.5 cc. after being mixed and incubated for 2 hours at 37.5° and left overnight in the ice box.

Serum	Car- bohy- drate	D lution * of carbohydrates (on ash-free basis)															
		80	90	100	120	140	160	180	200	220	240	260	280	300	320	340	360
Ra	Ra	++	++	++	++	++	++	++	++	++	++	+	+	+	+	±	±
	Rv	++	++	++	++	++	++	++	+	+	+	+	+	±	-	-	-
Rv	Ra	++	++	±	-	-	-	-	-	-	-	-	-	-	-	-	-
	Rv	++	++	++	++	+	+	±	±	-	-	-	-	-	-	-	-

\* Diluted on a basis of 1:80,000, 1:90,000, etc.



Table II records the reactions of the sera tested with the above carbohydrates.

The fact that sera Ra and Rv react less strongly with 520-C than with 520-B2a" is interesting in that Dr. Heidelberger and Dr. Menzel found that different rabbits immunized with the same vaccine may produce both antibodies (against high rotating as well as against low rotating carbohydrates), or may lack one of them.

TABLE II  
*Effect of Carbohydrates on Sera*

All serum and carbohydrate samples measured 0.5 cc. after being mixed and incubated for 2 hours at 37.5° and left overnight in the ice box; all carbohydrate dilutions were 1:50,000.

Carbohydrate	Serum	
	Ra	Rv
520-C	+±	+
520-B2a	+++	++±
520-B2a"	+±±	++±
520-B2-C	—	—
520-V1-C	+±±	+±
Salt solution	—	—

#### DISCUSSION

Our experiment was unique because the virulent and avirulent microorganisms used for the production of tuberculin were obtained from a single parent culture. The Ra microorganisms were of definite topography when planted on a Steenken and Smith medium for colony study. Inoculated in guinea pigs, they produced skin hypersensitivity but not progressive disease. The Rv microorganisms were also of definite topography when planted on a Steenken and Smith medium for colony study; they produced skin hypersensitivity, and, contrary to the Ra culture, produced progressive disease in guinea pigs.

When grown on the modified Proskauer and Beck medium, both variants were stable for 5 years as to virulence for guinea pigs and as to growth appearance.

Our yields of organisms were considerably less than those ob-

tained by Seibert and Long, because we used a different synthetic medium—a medium upon which we had been cultivating these microorganisms for a span of over 5 years without any noticeable change in virulence or growth characteristics.

Of the two variants we experienced a greater yield of Ra microorganisms, but a greater yield of PPD and carbohydrate from the tuberculin produced by Rv microorganisms. One would ordinarily expect a greater yield of PPD and carbohydrate from the variant with the greater growth. The anomaly is explained in a previous publication (5) from this Laboratory, in which we showed that Rv microorganisms are susceptible to lysis and Ra resistant to lysis. Therefore, we can assume that the difference in weight of the Rv microorganisms was due to a breakdown of some of the Rv microorganisms, and that the greater yield of PPD and carbohydrate in the Rv tuberculin resulted from broken down microorganisms.

We were unable to demonstrate any skin specificity in guinea pigs with PPD derived by ultrafiltration, although the more modern methods used by Seibert for separating protein fractions from tuberculins might do so.

To date, we have not tested the skin of any patients with either PPD or carbohydrates.

Our carbohydrates did contain some protein, but it was present in quantities too small to elicit skin reactions in sensitized guinea pigs.

According to Heidelberger, rabbits immunized with the same vaccine may produce one or both antibodies (against high rotating and low rotating carbohydrates). The difference between our carbohydrate tests with the goat antisera and Dr. Heidelberger's with the same sera against his carbohydrates may possibly be explained on the basis that his fractions were isolated from an H-37 culture of unknown virulence, and that the component present in one of our strains may have been entirely lacking in his material.

I wish to thank Dr. Michael Heidelberger, College of Physicians and Surgeons, Columbia University, and Dr. Florence B. Seibert of the Henry Phipps Institute, for their many helpful suggestions; also Dr. Gustave Meyer of the Rockefeller Institute for the nitrogen determination.

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# METABOLISM OF METHIONINE AND ITS DERIVATIVES WITH TISSUE SLICES\*

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Some time ago we reported that  $\alpha$ -keto- $\gamma$ -methiobutyric acid obtained from methionine by incubation with tissue slices is split by the action of acid or alkali into methyl mercaptan and an uncharacterized residue (1). About 80 per cent of the keto acid, estimated as the dinitrophenylhydrazone in aliquots of the solution, could be accounted for by the mercaptan isolated. This finding indicated that the linkage of the sulfur atom to the keto acid chain is broken, in contrast with the formation of homocysteine from methionine by the action of hot mineral acids. The readiness with which methionine is deaminized enzymatically (2) suggests that the keto acid may be its metabolically active derivative.

In the experiments presented in this paper we looked for products of enzymatic action other than the keto acid (3). In addition the metabolism of homocystine and homocysteine was investigated. Whereas the keto acid is the only product formed in significant amounts during enzymatic degradation of methionine by liver and kidney slices, the demethylated derivatives of methionine were not deaminized. Homocysteine yielded appreciable amounts of hydrogen sulfide.

## EXPERIMENTAL

The substrates used were recrystallized and ether-washed *dl*-methionine, *dl*-homocystine, and *dl*-homocysteine. For a reference sample of homocystine we are indebted to Dr. du Vigneaud.

\* This investigation has been supported by a grant from the Dazian Foundation for Medical Research.

The homocystine and homocysteine were prepared according to the procedure of Patterson and du Vigneaud (4) and also by the action of sodium upon methionine<sup>1</sup> in liquid ammonia (see also (5)). To permit the isolation of the metabolic products, the tissue slice experiments were carried out on a large scale in 250 ml. flasks according to the technique of Krebs, with phosphate buffer (6). The rat organs used were liver, kidney, and small intestine. The amounts of tissue (dry weight) and substrate are recorded in Table I. The flasks were shaken for 2 hours at 38°. The keto acid was determined gravimetrically in aliquots of the protein-free

TABLE I

*Balance Experiments on Methionine*

Liver and kidney slices in 50 ml. of Krebs' phosphate buffer were used.

Tissue	dl-Methionine	Tissue, dry	As <sub>2</sub> O <sub>3</sub>	Methionine converted to keto acid	Residual methionine recovered	
					As homo-cysteine	As CH <sub>3</sub> I
	mg.	mg.		per cent	per cent	per cent
Liver	447	400	+	7.4	96	98
	447	300	+	10.7	81	92
	149	500	—	4.0		93
	149	520	—	1.4	98	94
Kidney	298	200	+	22.5	92	93
	298	130	+	20.2	76	95
	149	215	—	19.6	94	95
	149	256	—	20.5	93	94
	149	160	+	30.4		

filtrate as the 2,4-dinitrophenylhydrazone which exists in a red and a yellow form, the latter being the more stable and less soluble. The sulfate formed was determined as barium sulfate (7). Residual methionine<sup>2</sup> was determined by the Baernstein procedure (8, 9) as volatile iodide and as homocysteine after the removal of the keto acid. With samples of pure methionine the recoveries of iodide corresponded to those reported by Kassell and Brand (9);

<sup>1</sup> The preparation of small amounts of homocysteine by the latter procedure which furnishes yields of about 60 per cent has proved convenient.

<sup>2</sup> We are indebted to Dr. Brand and Dr. Kassell for the use of an improved apparatus (unpublished) for the methionine determination.

the recoveries of homocysteine to those reported by du Vigneaud *et al.* (10). The sulfate found in the experiments with liver and kidney slices corresponded to 2 per cent of the methionine added, a finding in agreement with that of Pirie (11).

As expected (6), kidney slices form more keto acid than liver slices, the yield being increased by the addition of  $\text{As}_2\text{O}_3$ . Calculated on the basis of the volatile iodide, 93 to 98 per cent of the residual methionine could be recovered in the experiments with both liver slices and kidney slices. The recovery of homocysteine has not always been as satisfactory as that of volatile iodide. The blanks in the homocysteine determination corresponded to those reported by Kassell and Brand (9). A variation in the blank value of 0.2 ml. of diiodate corresponds to 0.3 mg. of methionine. Since we used aliquots corresponding to about 5 mg. of methionine, considerable deviations might be encountered. Therefore we do not feel justified in ascribing the discrepancies in the values of volatile iodide and homocysteine to a shift of the methyl group and an actual disappearance of homocysteine. For the detection of  $-\text{S}-\text{S}-$ ,  $\text{SH}$ , or thiolactone groups the nitroprusside reaction was employed. Pirie reported a "faint but definite" nitroprusside reaction in similar experiments. In numerous experiments in which liver, kidney, and intestine acted on methionine we found that the nitroprusside reactions never exceeded those given by controls without added methionine. ■

It is of interest that liver and kidney slices of a cystinuric dog deaminized 27 and 31 per cent respectively of added *dl*-methionine.<sup>3</sup>

#### DISCUSSION

Our experiments indicate that the only metabolic product of methionine, produced in significant amounts in the presence of tissue slices, is the corresponding keto acid. It has already been shown (12) that the keto acid is a metabolic product of methionine, since it was possible to isolate it as the dinitrophenylhydrazone from the urine of rats which were fed a methionine-fat-carbohydrate diet. These findings agree well with the evidence that unnatural *d*-methionine and *d*-homocysteine (with choline) support growth on a methionine-deficient diet (13, 14). Since

<sup>3</sup> Brand, E., Kassell, B., Waelsch, H., and Borek, E., unpublished experiments.

homocystine, together with choline, as the methyl donor, supports growth in place of methionine, it was suggested by du Vigneaud *et al.* that homocystine is methylated to methionine in the animal body (14). The reverse procedure, namely the scission into homocysteine and the methyl group, was assumed to be the main pathway of the metabolic breakdown. As another possibility the formation of the keto acid of methionine and subsequent splitting into the keto acid of homocysteine and the methyl group have been suggested by Patterson *et al.* (15). The labilization of the whole methiol group offers another pathway for the metabolism of the keto acid. It should be emphasized here that we do not believe that the mercaptan group is actually set free during the metabolism of methionine but regard the process rather as a simultaneous yielding of both the methyl group and the sulfur to two different acceptors.

In the presence of tissue slices no indication of the formation of homocysteine could be found. The occurrence of the keto acid as a metabolic product was established not only in the experiments with tissue slices presented in this paper but also by isolation after methionine was fed to rats (12). However, the formation of homocysteine in living animals cannot be excluded. Our experiments suggest merely that the metabolic breakdown can proceed normally by way of the keto acid which is labilized between the carbon chain and the methiol group. Homocystine and homocysteine are not deaminized by tissues slices but they are metabolized in the intact animal. The formation of hydrogen sulfide from homocysteine as from cysteine (16) by the action of tissue slices may point to a different pathway of metabolic breakdown of these compounds. Since with methionine no trace of hydrogen sulfide can be detected,<sup>4</sup> it seems unlikely that homocysteine can be formed as a primary product.

Added methionine increases the yield of creatine in tissue slice experiments in the presence of glycoeyamine (18). For the methylation of glycoeyamine only a small portion, about 2 per cent of the added methionine, is used. This corresponds approximately to the amount of sulfate formed from methionine in our experiments with liver slices. This agreement might indicate that the sulfur of the methionine is oxidized to sulfate if the methyl group

<sup>4</sup> A sample of glycyl-L-methionine, kindly provided by W. C. Hess (17) also showed no increase of the nitroprusside reaction over that in the control.

is accepted by another compound. The oxidation may occur only if no suitable thio group acceptor is available. In experiments now in progress we are testing the various intermediates of carbohydrate metabolism, such as phosphopyruvic acid, for their ability to serve as acceptors for the methionine sulfur.

## SUMMARY

The main product in the first stage of the metabolism of *dl*-methionine with tissue slices of kidney or liver is the corresponding keto acid. Sulfate is formed to an extent of 2 per cent of the added methionine. No increase of the nitroprusside reaction over that in controls could be found. Undeaminized methionine was recovered within the experimental error.

Homocystine and homocysteine are not deaminized by tissue slices. Homocysteine forms hydrogen sulfide.

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# THE SODIUM CHLORIDE LEVELS AND THE EFFECT OF SODIUM CHLORIDE ADMINISTRATION ON THE ABNORMAL MANIFESTATIONS ASSOCIATED WITH A DEFICIENCY OF THE FILTRATE FACTORS OF VITAMIN B IN RATS\*

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It has been reported and confirmed (1-3) that rats fed a diet deficient in the filtrate factors of vitamin B developed graying of the fur. Furthermore, under the same experimental conditions, certain pathological changes occurred in the adrenal cortex (4). The changes have consisted for the most part of hemorrhage, necrosis, and atrophy.

There has been ample evidence that the adrenal cortex was concerned with the metabolism of sodium and chloride (5, 6). In view of this, the question arose as to whether the manifestations associated with a deficiency of the filtrate factor in rats could be influenced by the amounts of sodium chloride in the diet. Furthermore, it seemed important to establish whether the changes produced in the adrenal cortex, as a result of the deficiency, were sufficient to affect the plasma level of chlorides.

## *Procedure*

Rats of the Long-Evans strain were used for the studies. The animals were bred in the laboratory and litter mates were used. The rats were started on the experimental diet when 4 weeks old.

The basal diet consisted of casein 22 per cent, sucrose 64 per cent, primex 9 per cent, cod liver oil 2 cc. per 100 gm., and a modi-

\* This research was aided by a grant from the Josiah Macy, Jr., Foundation.

fied salt mixture 5 per cent (this contained ferric, potassium, and magnesium citrate, calcium diphosphate, copper sulfate, and potassium iodide). 12.5 cc. of Galen B were added to 100 gm. of the diet for the control rats. The diets of the experimental rats were supplemented with thiamine chloride, pyridoxine, and riboflavin, so that each rat received daily about 15  $\gamma$  each of the first two and 45  $\gamma$  of riboflavin.

Three animals were kept in a cage and they were allowed to eat the diet *ad libitum*. The diet contained no sodium chloride. The sodium chloride was given in water and placed in a drinking bottle with a curved glass tube outlet from which the rats drank directly, so that none was lost by leakage. The rats on the low salt intake were given a solution containing 0.064 per cent NaCl. Three rats consumed about 50 cc. of the salt solution daily. The salt solution selected for use in the diets with the low salt intake contained a concentration of NaCl equal to that found in Galen B. Since Galen B served as the source of the complete vitamin B complex in the diet of the control rats, the dilute salt solution used made the intake of the experimental and control rats comparable.

The rats on the high salt intake were given a 0.9 per cent solution of NaCl. Three rats were allowed 100 cc. daily of the solution.

Two groups of control rats were included in the study, one group of three rats on the high NaCl intake and one group of three rats on the low NaCl intake. As a complete source of the vitamin B complex 125 cc. of Galen B per kilo were added to the basal diet of the control animals.

At the end of the experimental period, when graying of the fur had occurred, blood was withdrawn from the tail. The tail was heated over an electric light bulb for a few moments, a piece was clipped off, and the tail was immediately inserted under oil into a small centrifuge tube. For the determination of the serum chloride Hald's modification of Paterson's micromethod was employed (7). In addition the serum protein was determined by the falling drop method (8).

When the animals were sacrificed, both adrenals were removed and sectioned. The results of the pathological studies, including observations on other organs, will be reported in detail in a separate communication. The adrenal findings are summarized briefly in

Tables I and II. We are indebted to Dr. Irving Graef of the Department of Pathology for a report of the pathological findings.

### *Results*

In all, twenty-one rats were studied. Series C-1 consisted of nine rats, six of which were given the deficient diet plus the high salt intake. Three rats served as controls for this group and were given the experimental diet plus Galen B and the high salt intake. Series C-2 consisted of twelve rats. Nine rats were fed the deficient diet and the low salt intake. Three rats, serving as controls, were given the experimental diet plus Galen B. The only salt was that contained in the Galen B. The observations on all the animals were continued for an average period of 100 days.

The three control rats in *both* groups gained an average of 111 gm. during the period of observation. Their coats remained in excellent condition and there was no evidence of any graying. They were sacrificed at 99 and 110 days.

*Series C-2 on Low Salt Intake (Table I)*—The rats receiving the low salt intake failed to gain weight normally. At the end of the experimental period, the average weight was 83 gm. as compared with an average weight of 150 gm. in the three normal controls. Distinct and marked graying of the fur occurred in all but one animal in the experimental group. One animal died on the 53rd day of the experiment and in this rat the graying was obscured owing to a profound loss of fur. In the other rats the graying usually began over the head and back of the neck, spread uniformly down the sides of the body, and finally involved the entire body. In estimating the time of onset of the graying, we have reported the time when it was first observed and the time when graying had become pronounced. When first observed, a small patch of slightly gray fur was found over the head or back of the neck. The change in the color of the fur occurred about 10 days before graying became pronounced, by which time it had also spread over more of the body. As the experiment proceeded the fur over most of the body became silvery gray. In five of the nine rats the graying was severe. In three animals it was somewhat less intense and as previously mentioned in the rat which died on the 53rd day it was obscured, owing to the alopecia. The average time of onset of pronounced graying in the rats on the low salt diet was 43 days.

In one animal the graying was definite after 29 days and in one animal it was not distinct until the 62nd day. First graying of the fur, however, was observed in the rats on the low salt intake after an average period of 32 days. The range of the time of onset was from 29 to 47 days.

TABLE I

*Summary of Findings of Rats on Low Salt Intake. Series C-2*

0 = none, S. = severe, Ma. = marked, M. = moderate, Sl. = slight.

	Rat No.	Terminal serum Cl	Terminal serum protein	Onset of graying			Failure to gain weight	Degree of graying	Alopecia	Dermatitis	Superficial hemorrhages of skin	Adrenal changes*	
				1st noted day	Definite day	Day killed						Lipid depletion	Atrophy
		<i>m.eq. per l.</i>	<i>gm. per cent.</i>										
Filtrate factor-deficient	39	105				53	S.	?	S.	M.	0	M.	Sl.
	40	109	5.95	29	39	83	"	S.	"	S.	S.	"	
	41	108	4.66	29	29	96	M.	"	"	0	0	Sl.	Sl.
	42	(91)†	(6.15)†	39	46	100	Sl.	M.	Sl.	0	M.	"	0
	43	106	5.14	29	39	98	M.	"	S.	S.	0	M.	0
	44	96	5.17	39	47	99	"	S.	0	M.	M.	Ma.	0
	45	101	5.81	39	39	110	"	M.	M.	0	S.	Sl.	0
	46	100	5.48	29	39	110	"	S.	"	0	M.	0	0
	47	98	6.19	47	62	110	S.	"	S.	0	S.	Sl.	0
	48	99	5.99	0	0	110	0	0	0	0	0	0	0
Control	49	100	5.66	0	0	110	0	0	0	0	0	0	0
	50	98	5.92	0	0	99	0	0	0	0	0	Sl.	M. to S.

\* The adrenal pathology was limited to the juxtamedullary zone.

† Blood hemolyzed.

In addition, five of the animals on the low salt intake developed a severe alopecia. The fur was brittle and patches of it fell out, exposing areas of skin. In four rats there was a generalized dermatitis and in seven of the animals there were superficial hemorrhages over the feet.

The adrenal changes were confined to the juxtamedullary zone of the cortex. There was no evidence of hemorrhages in any of the

adrenals. The adrenals in seven of the animals showed from slight to marked lipid depletion. In only two of the adrenals was any atrophy of the cortical cells found.

The general appearance of the animals was poor, as is shown in Fig. 1. The rats on the low salt intake appeared to be in a poorer state of nutrition than the experimental rats on the high salt intake.

*Series C-1 on High Salt Intake (Table II)*—The experimental rats on the high salt intake also failed to gain weight, but the failure was not as pronounced as in the rats on the low salt diet. Interestingly enough, only three of the six rats developed graying of the



FIG. 1. Comparison of a control (Rat 57), a rat on the diet deficient in the filtrate factor plus the low salt intake (Rat 41), and a rat on the deficient diet and the high salt intake (Rat 54). The more profound graying of the fur in the rat on the low salt intake is quite evident and was typical of the group.

fur and in one rat it was always slight. The graying that did occur was first observed on the 75th day and did not become pronounced until about the 100th day. The onset of definite graying in the rats on the high salt intake was 55 days later than the onset in the rats on the low salt intake.

Alopecia was present to a slight or moderate degree in four of the rats on the high salt intake and four of the animals also showed some superficial hemorrhages over the feet. On pathological examination the adrenals of four rats showed a slight amount of lipid depletion and in one rat the depletion was moderate. Severe

atrophy of the cortical cells occurred in two others. A moderate amount of atrophy was observed in the adrenals of two of the control rats. The adrenal changes were again confined to the juxtamedullary zone of the cortex.

*Serum Chloride and Protein Values*.—The serum chloride values in the rats receiving the low salt intake were within normal limits. In milliequivalents per liter, the serum chloride varied from 96 to

TABLE II

*Summary of Findings of Rats on High Salt Intake. Series C-1*

0 = none, S. = severe, M. = moderate, Sl. = slight.

	Rat No.	Terminal serum Cl	Terminal serum protein	Onset of graying		Day killed	Failure to gain weight	Degree of graying	Alopecia	Superficial hemorrhages of skin	Adrenal changes*	
		m.eq. per l.	gm. per cent	1st noted day	Definite day						Lipid depletion	Atrophy
Experimental	51		7.14	75	100	110	M.	Sl.	Sl.	M.	Sl.	M.
	52	107	6.66	100	?	110	"	±	"	"	"	S.
	53	106	7.04	75	100	110	Sl.	S.	M.	S.	"	"
	54	105	4.56	0	0	96	M.	0	0	0	"	M.
												to S.
Control	55			0	0	96	"	0	0	Sl.	0	0
	56	102	5.44	0	0	99	Sl.	0	Sl.	0	M.	Sl.
	57	108	5.66	0	0	96	0	0	0	0	0	0
	58	99	6.94	0	0	110	0	0	0	0	0	M.
	59	97	6.02	0	0	110	0	0	0	0	Sl.	"

\* The adrenal pathology was limited to the juxtamedullary zone.

109. The serum protein varied from 4.66 to 6.19 gm. per cent in this group. In the three control rats, kept on a low salt intake, the serum chloride levels were 98, 99, and 100 milliequivalents per liter and the serum protein was quite uniform and averaged 5.86 gm. per cent. In the experimental rats on the high salt intake the terminal serum chloride values ranged from 102 to 107 milliequivalents per liter. The serum protein values varied from 4.6 to 7.1 gm. per cent. The serum chloride levels in the control animals in this group ranged from 97 to 108 milliequivalents per liter and the serum protein values ranged from 6.9 to 5.7 gm. per cent.

## DISCUSSION

Of the fifteen rats observed on diets deficient in the filtrate factors of the vitamin B complex, those in which the salt intake was kept very low developed pronounced graying of the fur. Furthermore the graying occurred much sooner and the incidence was greater in the rats on the low salt intake than in the rats on the high salt intake. In addition the rats on the low salt ration developed the other skin manifestations associated with this deficiency (2, 9), such as alopecia, dermatitis, and superficial hemorrhages of the skin. It seems clear that the amount of salt added to the filtrate factor-deficient diet influenced the incidence, extent, and degree of graying of the fur that occurred in the rats. Morgan (10) mentioned that "three dogs were placed on a salt-free . . . diet, and this complicated the effect of the vitamin deficiency in an unexpected way." No further reference was made to the animals in this report, so that it was not clear what the complications were.

The adrenal changes that were observed in the experimental animals on the low salt intake were mostly those of lipid depletion of the juxtamedullary zone of the cortex. In the experimental animals on the high salt ration, lipid depletion of the cortex also occurred and in addition a moderate to severe degree of atrophy was present in the adrenals of four rats.

Daft and Sebrell (4) have reported hemorrhage, necrosis, and atrophy of the adrenal cortex in rats kept on a diet deficient in the filtrate factors. Nicotinic acid and choline were added to the diets used in their experiments. The fact that neither hemorrhage nor necrosis was observed in the adrenals of the rats we have studied raises the question as to whether the more severe changes reported by other investigators may not have been due to the toxic effects of nicotinic acid or choline *in the absence of* the filtrate factors. The amount of salt added to the diet did not seem to protect the adrenal cortex from atrophy or lipid depletion.

In none of the rats was the extent of the adrenal pathology severe enough to influence the serum chloride values. We confined ourselves to the chloride determinations in these experiments, because the micromethod for sodium determinations is not entirely satisfactory. The sodium level in the plasma is also definitely influenced by the adrenal cortex hormone, but any significant change in the serum sodium is usually associated with changes in the



chloride level. It might well be that more pronounced pathological damage of the adrenal cortex would influence the level of chloride in the blood and experiments are in progress which we hope will answer this question. The fact that the terminal serum proteins were normal showed that no profound hemoconcentration had occurred in these animals.

#### SUMMARY

Fifteen rats of the Long-Evans strain were placed on diets deficient in the filtrate factor of the vitamin B complex. In nine of these animals the NaCl content of the diet was kept at a very low level. The other six animals were given large amounts of NaCl. Six control rats were studied on diets adequate in the entire vitamin B complex, three on a low salt intake and three on a high salt intake.

The rats on the low salt intake developed graying of the fur, on an average, 55 days sooner than did the animals on the high salt intake. Furthermore the graying was more pronounced and occurred in all but one of the nine rats.

Atrophy of the cells in the juxtamedullary zone of the adrenal cortex occurred to a greater extent in the animals on the high salt intake. The lipid depletion was more profound in the adrenal cortex of the rats on the low salt intake. In none of the animals were hemorrhages of the adrenal cortex observed.

The serum chloride levels, determined in all of the animals at the end of the experimental period, were within normal limits, as were the serum protein values. Obviously the pathological changes that did occur in the adrenal cortex of the rats were not sufficient to influence the chloride levels of the serum. The fact that the serum protein values were within normal limits indicated that hemoconcentration did not occur.

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# MICRODIFFUSION METHODS BASED ON THE BISULFITE REACTION

## I. THE DETERMINATION OF ACETONE\*

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A microdiffusion method for acetone, utilizing the Conway unit (1), has been recently described by Werch (2). The determination is based upon the diffusion of acetone from a blood or urine sample placed in the outer chamber of the unit into the central chamber, where a precipitate is formed with  $\text{HgSO}_4$  (Denigès' reagent). By noting the exact time which is required for the first appearance of the precipitate at a given temperature, the amount of acetone can be calculated with the aid of a nomogram. In the present author's experience with this method, duplicate determinations did not agree more closely than 20 to 30 per cent when a solution containing 20 mg. of acetone per 100 ml. of water was used.

In the present microdiffusion procedure, the acetone-binding action of  $\text{NaHSO}_3$  is used as the basis of a quantitative titration method. Klein (3) has shown that the bisulfite reaction can be used for the quantitative determination of acetone plus acetoacetic acid in blood filtrates free of sugar. The dissociation of the carbonyl-bisulfite compounds is repressed by using a large excess of  $\text{NaHSO}_3$  and lowering the temperature to  $5^\circ$ . After completion of the addition reaction, the excess of bisulfite is removed by concentrated iodine solution, and the solution is made alkaline with  $\text{Na}_2\text{HPO}_4$ , thereby dissociating the carbonyl-bisulfite compounds. The bisulfite thus liberated is then titrated with standard 0.005 N iodine solution. Most of the pyruvic acid normally present in blood is also determined by this method.

\* Aided by a grant from the McGregor Fund.

By modifying Klein's method to utilize the Conway diffusion unit, it is possible to determine acetone alone directly on whole blood or serum, as well as on urine. The acetone diffuses from the blood or urine in the outer chamber into bisulfite solution in the central chamber of the unit, where it is determined according to the principles described in the preceding paragraph.

In addition to simplicity of operation, the present method offers greater specificity and accuracy than the older and more complex procedures which usually depend upon the precipitation of the acetone as a mercury complex. The bisulfite solution in the central chamber of the unit is separated from the possible interfering substances present in the blood or urine in the outer chamber, and only volatile substances which combine reversibly with  $\text{NaHSO}_3$  are measured. By using a number of units it is possible to run several determinations simultaneously.

### *Procedure*

Blood samples are collected in tubes which contain oxalate or citrate and are tightly fitted with rubber stoppers. The blood (or urine) can stand for a number of hours in the ice box without appreciable change in the acetone concentration.

2 ml. of approximately 0.15 M  $\text{NaHSO}_3$ <sup>1</sup> are introduced into the central chamber, and 0.5 ml. of 2N  $\text{H}_2\text{SO}_4$ <sup>1,2</sup> into the outer chamber of a Conway diffusion unit. A cover plate suitably greased with stop-cock lubricant is placed over the unit, leaving a narrow opening for the insertion of a pipette. A 1 or 2 ml. sample of the blood or urine is then quickly introduced into the outer chamber, and the cover is immediately slid over to seal the unit completely. If blood is being analyzed, the unit is rotated at once to mix the blood and the acid.

After 6 hours or more at room temperature (about 25°),<sup>3</sup> the unit

<sup>1</sup> The volume need not be precisely measured.

<sup>2</sup> Acid must be used to prevent loss of bisulfite from the central solution by diffusion of  $\text{SO}_2$  into the outer chamber, where the  $\text{SO}_2$  apparently reduces the hemoglobin of the blood. The acid is omitted when urine is analyzed.

<sup>3</sup> By incubating the units at 40–50°, the time required for complete diffusion is reduced to about 3 hours. It is advisable to use a lubricant of higher melting point, made by melting 2 parts of vaseline with 1 part of paraffin wax, for sealing the units at these temperatures.

is cooled for at least half an hour in a shallow dish of water containing ice cubes. The cover plate is removed, and a small drop of starch solution is added to the  $\text{NaHSO}_3$  solution in the central chamber. By means of a fine glass stirring rod, the large excess of  $\text{NaHSO}_3$  is removed with 1 N iodine solution added very slowly, dropwise from a burette. As the end-point is neared, small fractions of drops of iodine are transferred from the burette tip to the solution with the tip of the stirring rod.

When a permanent light purple color is reached, the unit is removed from the ice bath and warmed to room temperature. Then about 0.3 to 0.4 gm. of powdered  $\text{Na}_2\text{HPO}_4$  is added to the central solution, and the latter is stirred well. The  $\text{NaHSO}_3$  which is liberated from combination with the acetone is titrated with standard 0.005 N iodine solution to the same end-point as before.

Owing to the small volume of the solution being titrated, a definite color change at the end-point is produced by about 0.005 ml. of the standard iodine. Since 1 ml. of 0.005 N iodine is equivalent to 0.145 mg. of acetone, the mg. of acetone per 100 ml. of blood or urine can be calculated at once. The volume of the first titration with 1 N iodine obviously need not be recorded.

*Rates of Absorption of Acetone*—Table I shows that acetone is absorbed at about equal rates from water, urine, or blood. The slight differences in the absorption rates may be partly due to differences in room temperature and to differences in the volumes of fluid in the outer chambers. The recoveries after 5 to 6 hours diffusion are seen to be 99 per cent complete for pure water and for urine, and 96 and 98 per cent respectively for bloods containing low and high levels of acetone. The blood and urine values were corrected for the very small quantities of acetone normally present. Duplicate determinations with diffusions of 6 hours or longer usually agreed to within 2 to 3 per cent when the titration amounted to about 1 ml. of 0.005 N iodine solution.

*Comparison with Klein's Bisulfite Method*—Analyses on 2 ml. samples of normal blood containing 15.5 mg. of (added) acetone per 100 ml. gave the following recoveries after a 6 hour diffusion period, 96, 98, 98, 99 per cent. The recoveries by Klein's method (3), when 1 ml. samples of the same blood solution were used, were 90, 91, 95, 97, 102 per cent. The lower average value (95 per cent) of the latter series probably reflects slight losses of acetone during

the removal of proteins and sugar. Klein also found low values for this level of acetone concentration.

*Normal Blood and Urine Levels*—The urine and blood of six normal individuals contained almost negligible amounts of acetone. The values ranged from 0.1 to 0.5 mg. of acetone per 100 ml.

*Blood Acetone Levels in Controlled Diabetics*—Analyses were made on 2 ml. samples of the blood of eleven patients of the Detroit Receiving Hospital. The values ranged from 0.5 to 2.0 mg. of acetone

TABLE I

*Rate of Recovery of Acetone Added to Pure Water, Normal Urine, and Normal Blood*

The solutions were prepared by adding to the water, urine, or blood suitable volumes of a relatively concentrated acetone solution. The latter was standardized by the iodoform method (4). 2 ml. aliquots of the solutions were analyzed, except for the data of the last column, for which 1 ml. samples were used.

Time of diffusion	Rate of absorption			
	8.0 mg. acetone per 100 ml. water	7.8 mg. acetone per 100 ml. urine	3.9 mg. acetone per 100 ml. blood	39 mg. acetone per 100 ml. blood
hrs.	per cent	per cent	per cent	per cent
1.0	60	64	72	65
1.5	70			
2.0		82	83	81
2.5	81			
3.0			87	89
3.5	87			
4.0	92	96	93	95
5.0	97	99	95	98
6.0	99	98	97	97
7.0	101		96	98

per 100 ml. of blood. The average value, 1.1, is 2 to 3 times greater than the average found for normal blood. Duplicate determinations agreed to within about 10 per cent in this range of low acetone values.

*Blood and Urine Levels in Diabetic Coma*—To illustrate the application of the present method to samples containing higher concentrations of acetone, measurements were made on a patient who was initially in diabetic coma. Duplicate analyses on 1 ml. samples gave values of 29.9 and 30.7 mg. of acetone per 100 ml. of

blood, and 27.6 and 28.1 mg. of acetone per 100 ml. of urine. 24 hours later, when the patient had recovered from the coma, the acetone values obtained for duplicate analyses were 6.9 and 7.1 mg. per 100 ml. of blood, and 10.2 and 10.5 mg. of acetone per 100 ml. of urine.

*Rate of Disappearance of Acetone from Dog Blood Following Intravenous Injection*—Chaikoff and Soskin (5) have followed the level of acetone bodies in the blood of dogs after intravenous injection of the sodium salt of acetoacetic acid at a level of 1 gm. per kilo of body weight. It was thought of interest to make a series

TABLE II

*Rate of Disappearance of Acetone from Blood Following Intravenous Injection*

The analyses were performed on 0.5 ml. aliquots of the blood samples collected during the first 6 hours, and on 1 ml. aliquots of the last three samples.

Time after acetone injection	Acetone found in blood
hrs.	mg. per 100 ml.
0.1	66.0
0.25	57.0
1	52.5
3	40.5
6	34.0
12	24.0
25	7.0
49	1.0

of similar measurements with acetone, which would illustrate the use of the present method over a wide range of acetone concentrations.

A normal, 10 kilo dog, whose blood initially contained less than 0.5 mg. of acetone per 100 ml., was given by intravenous injection 65 ml. of an isotonic saline solution which contained 6.25 gm. of acetone per 100 ml.<sup>4</sup> Blood samples were taken at different times, and the acetone concentrations determined.

Assuming a blood volume of about 1 liter, the 4 gm. of acetone injected produced an initial level of approximately 400 mg. of acetone per 100 ml. of blood. Table II shows that more than 80 per

<sup>4</sup> There were no indications of injury to the animal, or even discomfort, due to the acetone injection.



cent of this acetone was removed from the blood almost immediately. The level of acetone is seen to fall most rapidly during the first 2 or 3 hours and then decrease at a fairly slow and uniform rate. These results are similar to those of Chaikoff and Soskin for acetone bodies. These investigators concluded that acetoacetic acid is rapidly utilized by muscle.

#### SUMMARY

A sensitive microdiffusion procedure for the quantitative determination of acetone in blood and urine has been developed, based on the use of the bisulfite reaction. The method appears to be highly specific in that only volatile substances are measured which can diffuse as gases from whole blood or urine into  $\text{NaHSO}_3$  solution, forming reversibly dissociable compounds with the latter. The solutions to be analyzed need not be free from protein and sugar.

Acetone added to pure water, blood, or urine is quantitatively recovered after diffusion periods of 5 to 6 hours at room temperature or 3 hours at  $40\text{--}50^\circ$ . The method is accurate to about 2 to 3 per cent for acetone levels of 10 to 30 mg. per 100 ml.

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# GROWTH INHIBITION BY N-( $\alpha,\gamma$ -DIHYDROXY- $\beta,\beta$ -DIMETHYLBUTYRYL)TAURINE AND ITS REVERSAL BY PANTOTHENIC ACID

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In a recent note to this *Journal* (1) the condensation of  $\alpha$ -hydroxy- $\beta,\beta$ -dimethyl- $\gamma$ -butyrolactone with the sodium salt of taurine was described. The product inhibited growth of *Lactobacillus arabinosus* 17-5. This inhibition did not appear when sufficient pantothenic acid was added to the medium; the level of pantothenic acid necessary to insure growth of the test organism increased as the amount of condensation product in the medium increased. The results suggested a competition between pantothenic acid and its sulfonic acid analogue for certain surfaces on or in the bacterial cell. The "toxic" action of the sulfonic acid thus appeared due only to its action in screening the essential pantothenic acid away from its site of action.

The sodium and barium salts of the sulfonic acid have now been prepared in pure form and their action on a variety of microorganisms investigated. Since it is not planned to investigate the problem further, the results obtained are presented below.

## EXPERIMENTAL

*Sodium Salt of N-( $\alpha,\gamma$ -Dihydroxy- $\beta,\beta$ -Dimethylbutyryl)Taurine*  
—5 gm. of *dl*- $\alpha$ -hydroxy- $\beta,\beta$ -dimethyl- $\gamma$ -butyrolactone were melted in the oven at 120°. To the melt were added 5.7 gm. of the dry, powdered sodium salt of taurine. The mixture, which gradually became homogeneous, was stirred at intervals during 5 hours, then cooled. Amino nitrogen determinations (Van Slyke) on this product showed the disappearance of 80 per cent of the free amino nitrogen.

2.9 gm. of the solid, brittle product were powdered and dis-

solved by heating in 100 cc. of absolute ethanol. The solution was filtered hot from a small amount of insoluble material. On cooling overnight the sodium salt of the sulfonic acid separated as a white, amorphous precipitate, which was filtered off. On concentration of the mother liquors to 40 cc. and cooling, more of the product was obtained. The combined precipitates (2 gm.) of the sodium salt were redissolved in 50 cc. of hot alcohol, and separated by cooling as before. The product (1.3 gm.) was dried first over calcium chloride and finally over phosphorus pentoxide at 100°.

$C_8H_{16}O_6NSNa$ .      Calculated. N 5.05, Na 8.30  
                                  Found.      " 5.08, " 8.19

*Barium Salt of N-( $\alpha,\gamma$ -Dihydroxy- $\beta,\beta$ -Dimethylbutyryl)Taurine*—To 7.8 gm. of *dl*-lactone at 145° were added 11.2 gm. of the dry, powdered barium salt of taurine. The mixture was heated at 145–150° for 1 hour, then held at 135–140° for 2 hours. The viscous, yellowish mass set to a brittle solid.

4.0 gm. of the powdered salt were dissolved in 100 cc. of methanol, in which it is very soluble. After concentration to 40 cc., the solution was cooled and filtered. The barium salt was precipitated by addition of 5 volumes of acetone, filtered out, and reprecipitated twice in the same manner. The slightly yellow product was dissolved in water, heated for a short time with decolorizing carbon, filtered, then evaporated to dryness *in vacuo*. The residue (2.1 gm.) was dried *in vacuo* at 100° over phosphorus pentoxide.

$(C_8H_{16}O_6NS)_2Ba$ .      Calculated. N 4.34, Ba 21.27  
                                  Found.      " 4.27, " 21.29

*Effect of Sulfonic Acid on Bacteria*—The activity of all products was determined with *Lactobacillus arabinosus* 17-5. The organism was grown under the conditions and in the medium described by Snell and Wright (2) for the assay of nicotinic acid, except that nicotinic acid was added in excess (2  $\gamma$  per 10 cc.), and pantothenic acid added in varying quantities. The extent of growth was measured in all cases after 24 hours incubation by comparing turbidities quantitatively in the turbidimeter (3), which was calibrated by use of a cell suspension of the organism in question.

Results of tests on the pure salts showed them to have almost the same activity as the unpurified reaction products, indicating essentially quantitative yields in the condensation. Detailed data obtained with the crude sodium salt described above are given in Table I. From the data it is evident (a) that the sulfonic acid analogue of pantothenic acid has no growth-promoting effect by itself, but rather inhibits growth; (b) that the level required to inhibit growth increases as the amount of calcium pantothenate is increased; (c) even large amounts of the product are non-

TABLE I

*Effect of Crude Sodium Salt of N-( $\alpha,\gamma$ -Dihydroxy- $\beta,\beta$ -Dimethylbutyryl)-Taurine on Growth of *Lactobacillus arabinosus**

Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells	Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.	$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.
0.0	0	0.1	0.8	100	10.0
0.2	0	5.1	0.8	300	5.3
0.4	0	9.2	0.8	1,000	0.7
0.8	0	9.8	0.8	10,000	0.2
0.0	100	0.0	3.0	1,000	7.2
0.2	10	4.9	10.0	1,000	10.0
0.2	30	4.2	10.0	10,000	1.2
0.2	100	2.2	30.0	10,000	5.0
0.2	300	0.3	100.0	10,000	10.2
0.4	100	5.8	0.2	100*	3.8
0.4	300	1.6	0.2	1,000*	9.2
0.4	1000	0.3	0.2	10,000*	12.8

\* To these tubes, not the condensation product, but the indicated amounts of both taurine and the *dl*-lactone were added.

toxic if sufficient pantothenic acid is added; and (d) mixtures of taurine and lactone have no such inhibiting action, but rather stimulate growth somewhat when the level of pantothenic acid in the medium is suboptimal. Separate experiments showed this stimulation to be due to the lactone alone, which appears to be somewhat available for growth of this organism at these high concentrations. Woolley (4) has previously described a strain of hemolytic streptococcus which can utilize the lactone moiety of pantothenic acid for growth.

From the known fact that only dextrorotatory pantothenic acid

(from the levorotatory lactone) possesses growth-promoting activity for lactic acid bacteria (5) it appears probable that only the sulfonic acid derived from the levorotatory lactone would possess growth-inhibiting properties. While no attempt has been made to separate enantiomorphs, condensations have been run with both the (+)- and (-)-lactones,<sup>1</sup> and the sodium salts obtained purified as described above and tested. Data are given in Table II. The sulfonic acid from the (-)-lactone is about 10 times as active as that from the (+)-lactone, despite the racemization which must have occurred with both forms in the alkaline

TABLE II

*Comparative Activities of Condensation Products Derived from (+)- and (-)-Lactones*

The test organism was *Lactobacillus arabinosus*.

Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells		
		Sodium salt from dl-lactone	Sodium salt from (-)-lactone	Sodium salt from (+)-lactone
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.	mg. per 10 cc.	mg. per 10 cc.
0	0	0.0	0.0	0.0
0.3	0	13.8	13.8	13.8
0.3	10	13.6	12.7	13.8
0.3	30	10.0	6.8	13.8
0.3	100	5.0	3.5	13.0
0.3	300	2.6	0.0	7.9
0.3	1000	0.0	0.0	1.3
30.0	1000	13.8	13.8	13.8

melt at the elevated temperatures employed. This indicates clearly that the "toxic" action of the sulfonic acid is due chiefly, if not entirely, to the component part derived from the (-)-lactone. Thus the growth inhibition by the condensation product shows the same configurational specificity as does growth promotion by pantothenic acid. In further experiments the sodium salt derived from the (-)-lactone has been used.

In Table III are given results obtained with other organisms. *Lactobacillus arabinosus* is included for comparison. All of the organisms listed with the exception of *Escherichia coli* require

<sup>1</sup> Kindly furnished by Merck and Company, Inc.

pantothenic acid for growth (7); yet their sensitivity to growth inhibition by the sulfonic acid analogue varies greatly. *Lactobacillus arabinosus* is the most sensitive of all organisms tested, while the very similar *Lactobacillus pentosus* is among the most resistant. *Escherichia coli* is unaffected by the condensation product. Separate tests on *Staphylococcus aureus*, *Shigella para-*

TABLE III

*Effect of Sulfonic Acid Analogue of Pantothenic Acid on Other Species of Bacteria*

Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells					
		<i>Lactobacillus arabinosus</i> 17-5	<i>Lactobacillus pentosus</i> 124-2	<i>Streptococcus* lactis</i> R	<i>Propionibacterium* pentosaceum</i> P-11	<i>Leuconostoc mesenteroides</i> P-60	<i>Escherichia† coli</i>
$\gamma$ per 10 cc.	mg. per 10 cc.	mg. per 10 cc.	mg. per 10 cc.	mg. per 10 cc.	mg. per 10 cc.	mg. per 10 cc.	mg. per 10 cc.
0	0	0.05	0.9	0.9	1.1	0.9	4.8
0.3	0	13.8	9.9	4.2	15.0	4.3	
0.3	0.3	0.0	9.9	2.7	3.7	4.2	4.8
0.3	1.0	0.0	9.9	0.8	0.3	4.3	4.8
0.3	3.0	0.0	7.2	0.1	0.0	4.1	4.2
0.3	10.0		2.1	0.0	0.0	3.0	4.8
0.3	50.0		0.1	0.0	0.0	0.2	5.5
30.0	10.0	3.7	10.0	4.0	20.5	5.0	5.1
100.0	10.0	13.8	10.2	3.5	30.0	4.9	5.5
30.0	50.0		13.6	4.1	5.0	5.0	6.8
100.0	50.0		13.6	3.5	16.3	5.0	6.1

\* For these organisms 100  $\gamma$  of thymine were added per 10 cc. of medium (6). *Propionibacterium pentosaceum* was incubated for 48 hours, the remainder of the organisms for 24 hours, before turbidity measurements were made.

† No pantothenic acid was added for these organisms except for the results shown in the last four entries of the column.

*dysenteriae*, and *Brucella abortus* likewise failed to reveal inhibition in concentrations of the condensate up to 1.0 mg. per 10 cc. of medium.

*Effect of Sulfonic Acid on Yeast*—An interesting case is presented by yeast, which requires pantothenic acid for rapid and extensive growth, but which can utilize  $\beta$ -alanine for the same purpose. Tests were made with the Gebrüder Mayer strain of *Saccharomyces*

*cerevisiae* on two different media. In Table IV are data secured on the asparagine medium described by Williams and Saunders

TABLE IV

*Effect of Sulfonic Acid Analogue of Pantothenic Acid on Yeast Growth*

Incubation period, 16 hours; seeding, 0.04 mg. of moist cells per 10 cc. of medium; medium, Williams and Saunders (8).

Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells	Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.	$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.
0.0	0.0	0.12	0.0	3,000	0.0
0.01	0.0	0.30	0.1	3,000	0.12
0.02	0.0	0.65	0.3	3,000	0.50
0.03	0.0	1.0	1.0	3,000	1.10
0.0	1000	0.05	0.0	10,000	0.00
0.03	1000	0.20	0.3	10,000	0.05
0.05	1000	0.30	1.0	10,000	0.36
0.10	1000	0.70	3.0	10,000	1.30
0.30	1000	1.00			

TABLE V

*Effect of Sulfonic Acid Analogue of Pantothenic Acid and of  $\beta$ -Alanine on Yeast Growth*

Incubation period, 16 hours; seeding, 0.02 mg. of moist cells per 10 cc. of medium; medium, Snell *et al.* (9).

Calcium pantothenate	Sodium salt of sulfonic acid	Moist cells	$\beta$ -Alanine	Sodium salt of sulfonic acid	Moist cells
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.	$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per 10 cc.
0.0	0	0.3	0.0	0	0.03
0.05	0	0.6	0.3	0	2.8
0.10	0	1.7	0.5	0	5.5
0.20	0	6.2	1.0	0	6.6
0.50	0	6.6	3.0	0	6.6
0.5	1,000	2.9	0.3	1,000	3.0
0.5	3,000	0.8	0.3	10,000	3.0
0.5	5,000	0.4	0.5	5,000	5.5
0.5	10,000	0.3	0.5	10,000	5.7
30.0	10,000	6.6	0.3	10,000*	3.5

\* 10,000  $\gamma$  of taurine alone were added to this tube.

(8), which is unsupplemented with other nutritives. Progressively larger amounts of pantothenic acid are required to produce the

same growth response as the amount of sulfonic acid in the medium is increased. Thus with 10 mg. of the sulfonic acid present, approximately 3.0  $\gamma$  of calcium pantothenate are required to produce the same growth effect as is produced by 0.05  $\gamma$  in the base medium alone.

In further experiments the base medium used by Snell *et al.* (9) for biotin assay was modified by addition of 0.0002  $\gamma$  of biotin (concentrate) per 10 cc. of medium and omission of the  $\beta$ -alanine. Thiamine and pyridoxine were added as usual. On this medium the response to added pantothenic acid (or  $\beta$ -alanine) is much greater than on unsupplemented media, but occurs at an entirely different range of concentration. The data are given in Table V. Here again, growth induced by pantothenic acid is inhibited by the sulfonic acid analogue, but the inhibition is reversed by additional pantothenic acid. In striking contrast, growth induced by  $\beta$ -alanine is not inhibited by the sulfonic acid, although it has been assumed (10) that  $\beta$ -alanine served only as a precursor for pantothenic acid in stimulating yeast growth. This observation would, under proper conditions, permit a biological test for  $\beta$ -alanine to be carried out in the presence of pantothenic acid. Taurine likewise does not affect the growth-promoting action of  $\beta$ -alanine.

#### DISCUSSION

The data presented can all be explained on the assumption that the physiologically inactive sulfonic acid is structurally so similar to pantothenic acid that it interferes with the metabolism of the latter. McIlwain (11) has shown pyridine-3-sulfonic acid and its amide to interfere in nicotinic acid metabolism by microorganisms in this same manner, an observation which suggested the present work. With organisms which synthesize their own pantothenic acid, no toxic effect of the sulfonic acid has yet been observed. Similarly, when the organism can utilize  $\beta$ -alanine in place of pantothenic acid, no growth inhibition is apparent. No explanation can yet be given for the marked differences in sensitivity to the sulfonic acid of organisms which require preformed pantothenic acid. Two possibilities are (a) differences in cellular permeability to the sulfonic acid, and (b) quantitative or qualitative differences in pantothenic acid metabolism.



## SUMMARY

The preparation of the sodium and barium salts of N-( $\alpha$ , $\gamma$ -dihydroxy- $\beta$ , $\beta$ -dimethylbutyryl)taurine is described. These products, when present in sufficient amount, inhibit the growth of all organisms tested which require preformed pantothenic acid, although there are marked differences in sensitivity of various organisms to the substance. In all cases the inhibition can be overcome by supplying additional pantothenic acid. Growth inhibition has not been observed in organisms which synthesize their own pantothenic acid, either completely or from added  $\beta$ -alanine, although the number of such cases tested is limited.

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# A METHOD OF ALLOWING FOR THE INFLUENCE OF DIFFUSION IN MANOMETRIC MEASUREMENTS OF CERTAIN RAPID BIOCHEMICAL REACTIONS

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Manometric methods are widely used for measuring the rate of oxygen uptake and carbon dioxide output by enzyme solutions and tissue suspensions, and do indeed give the true chemical rates of such processes if these are slow compared with the rate of diffusion between the gas and liquid phases. With faster processes diffusion also becomes a limiting factor and the manometric readings then vary with the speed of shaking of the vessel and the relative volumes of the gas and liquid phases (Dixon and Elliott, 1930). If these conditions are kept constant, the observed rate should, in the case of a given process, be a definite function of the diffusion and chemical reaction velocities, and it might be possible to deduce from the observed over-all rate that due to the chemical reaction alone. If so, the manometric method could then be extended to faster biochemical processes than could previously be followed by its aid. The matter has lately been brought to a head by the need of studying the kinetics of  $\text{CO}_2$  uptake and output by buffer solutions, in the presence of carbonic anhydrase, at far higher rates than those for which the usual manometry was hitherto available. A procedure for calculating the true rates of chemical reactions in the body of the liquid has been worked out, and has been shown to be valid not only for carbonic anhydrase but also for  $\text{O}_2$  evolution from catalase solutions. The treatment is based on the stationary liquid film theory of physical chemists, which, though widely used for inorganic processes such as  $\text{CO}_2$  uptake by  $\text{Na}_2\text{CO}_3$  solutions, does not seem to have been used before in biochemical reactions, to which it may well have other applications besides the cases considered in this paper.

## STATIONARY FILM THEORY OF GAS-LIQUID INTERCHANGE

When a gas and a liquid are stirred or shaken together, it is assumed that the main bulks of the gas and liquid phases are infinitely well stirred but that on either side of the interface there are stationary films of gas and liquid, diffusion through which governs the rate of the interchange process (see reviews by Taylor (1930), Sherwood (1937)). In the case of gases of poor and moderate solubility, such as  $O_2$  and  $CO_2$ , the gas films can be neglected and the diffusion rate is then determined solely by the area and thickness of the liquid stationary film. The thickness of the liquid films varies from 0.001 to 0.04 cm., according to the speed of stirring or shaking. It is difficult to picture the physical forces responsible for maintaining such films many thousands of molecules thick, but their tangible existence seems to be shown beyond doubt by the direct optical observations of Davis and Crandall (1930).

*Simple Physical Solution of Gas*—When solution of the gas occurs without any chemical reaction, the stationary film theory states that

$$\text{Rate of gas uptake} = \frac{D_i}{\delta} A (c_i - c_L) \quad (1)$$

where  $D_i$  = diffusion coefficient of the dissolved gas

$\delta$  = thickness of the stationary film

$A$  = area of the stationary film

$c_i$  = concentration of the dissolved gas at the outer surface of the stationary film =  $\alpha p_i$

$\alpha$  = solubility coefficient of the gas in the liquid

$p_i$  = pressure of the gas in atmospheres

$c_L$  = average concentration of the dissolved gas in the bulk of the liquid phase

Equation 1 also holds good for liberation of dissolved gas from liquid. It has already been verified by numerous observers but, since its validity and application are crucial for the present paper, we have made further tests by the boat-manometric method (described by Roughton and Booth (1938)). In one such experiment 4.2 cc. of water were shaken smoothly at 290 times per minute with a 59 cc. gas phase containing  $CO_2$  at about 5 per cent atmosphere and the  $CO_2$  uptake followed manometrically. The results are plotted in Fig. 1, A.

- If  $p_0$  = initial  $\text{CO}_2$  pressure in the gas phase (in atmospheres)  
 $p_\infty$  =  $\text{CO}_2$  pressure in the gas phase when equilibrium with the liquid is reached  
 $c_\infty$  =  $\text{CO}_2$  concentration in the liquid at equilibrium (measured in cc. of  $\text{CO}_2$  per cc. of liquid)  
 $V_g$  = volume of gas phase in cc.  
 $V_L$  = volume of liquid in cc.  
 $G$  = total amount of  $\text{CO}_2$  in the gas and liquid phases

then from Equation 1 it follows that the rate of  $\text{CO}_2$  uptake in cc. per second =

$$-V_g \frac{dp_i}{dt} = -\frac{V_g}{\alpha} \frac{dc_i}{dt} = V_L \frac{dc_L}{dt} = \frac{D}{\delta} A (c_i - c_L) \quad (2)$$

Now

$$\frac{V_g c_i}{\alpha} + V_L c_L = G = \left( \frac{V_g}{\alpha} + V_L \right) c_\infty \quad (3)$$

Therefore

$$c_i - c_L = (c_i - c_\infty) \left( 1 + \frac{V_g}{\alpha V_L} \right) \quad (4)$$

From Equations 2 and 4 it follows that

$$-V_g \frac{dp_i}{dt} = \frac{D}{\delta} A (p_i - p_\infty) \left( \alpha + \frac{V_g}{V_L} \right) \quad (5)$$

Integrating, we have

$$\log \frac{p_0 - p_\infty}{p_i - p_\infty} = \frac{D}{\delta} A \left( \frac{\alpha}{V_g} + \frac{1}{V_L} \right) t \quad (6)$$

Log  $(p_i - p_\infty)$  plotted against time should therefore give a straight line of slope equal to  $DA(\alpha/V_g + 1/V_L)/\delta$ . Fig 1, B shows that this is so and the value of  $DA/\delta$  in the above units comes out at 0.44.

*Solution of Gas Accompanied by Chemical Reaction with Non-Volatile Solute*—In this case the solute also penetrates into the stationary film and reacts there with the dissolved gas as well as in the body of the liquid, so that conditions become very complex. If the chemical rate is very fast compared with diffusion and is

irreversible, then if various simplifying assumptions are made (see Davis and Crandall (1930)) the initial rate of gas uptake =

$$\frac{D_1 c_1 + D_2 c_2}{\delta} \quad (7)$$

where  $D_1$  = diffusion coefficient of the reacting solute

$c_1$  = concentration of the reacting solute in the body of the liquid

Equation 7 has been roughly verified in a few cases in which the reaction products do not alter conditions by their effect on

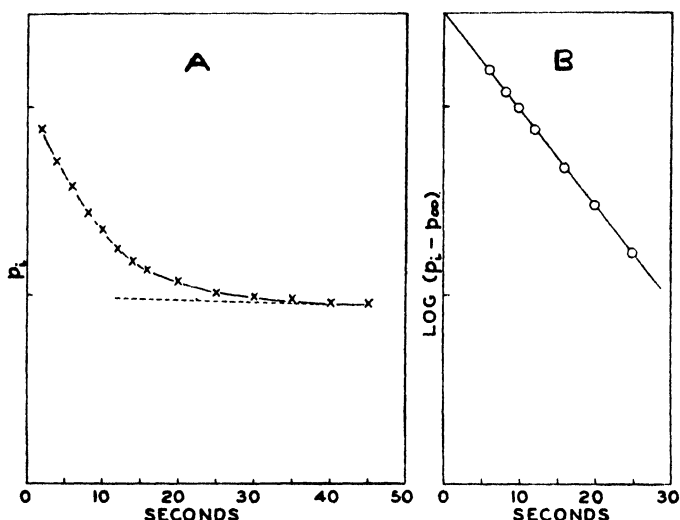


FIG. 1. Rate of uptake of  $\text{CO}_2$  by water. A = pressure of  $\text{CO}_2$  in gas phase *versus* time; B = test of Equation 6.

(a) the solubility of the gas, the diffusivity of the solutes, and the apparent thickness of the films, (b) the convection due to the heat produced in the films by the reactions.

A good qualitative example of the theory underlying Equation 7 is furnished by Dixon and Elliott's observation that at the usual rate of shaking (about 120 round trips per minute) the rate of oxygen uptake from air by concentrated yeast suspensions in the Barcroft apparatus is far less than by alkaline pyrogallol solutions. In the yeast suspensions  $D_1$  and  $c_1$  are both negligible compared with  $D_2$  and  $c_2$ , whereas in the pyrogallol solutions, in the

concentrations used,  $c_s$  is 20 or more times greater than  $c_i$  and  $D_s$  is probably not less than half of  $D_i$ . The  $D_s c_s$  term in Equation 7 should therefore be much greater than  $D_i c_i$  and the rate of  $O_2$  uptake should be correspondingly exalted. A quantitative test of Equation 7 is not, however, possible in this case, since the rate of reaction of  $O_2$  with pyrogallol is not "instantaneous" compared with the diffusion rates.

#### CALCULATION OF EFFECT OF DIFFUSION IN GAS-ENZYME REACTIONS

Enzymes, being proteins, have diffusion coefficients which are only about 1 to 5 per cent of those of dissolved  $O_2$  or  $CO_2$ ; the concentration of the enzyme is, as a rule, even lower relative to that of the dissolved gas. The  $D_s c_s$  term in Equation 7 is thus negligible in comparison with  $D_i c_i$ , and it therefore seems justifiable to disregard the chemical reactions within the stationary film, and, from the diffusion view-point, to treat the latter as equivalent to pure solvent. This simplifying assumption not only seems a priori sound, but also leads to good agreement of theory and experiment.

#### *Uptake Processes*

*Hydration of  $CO_2$  in Presence of Carbonic Anhydrase*—According to the Michaelis theory the true rate of this reaction equals

$$\frac{k_{su} E c_L}{c_L + K_{mu}} \quad (8)$$

where  $E$  = concentration of enzyme

$K_{mu}$  = Michaelis' constant of enzyme

$k_{su}$  = velocity constant for dissociation of the enzyme-substrate complex

The validity of Equation 8 for carbonic anhydrase has been confirmed by Roughton and Booth (unpublished). If  $c_L$  is small compared with  $K_{mu}$ ,  $R$  tends to the value  $k_{su} E c_L / K_{mu}$  and at fixed enzyme concentration the reaction is thus practically unimolecular with respect to dissolved  $CO_2$ . This is the simplest case to work out and test. The solution is as follows: when the steady state is reached, the rate of diffusion of  $CO_2$  through the stationary film equals its rate of combination in the body of the liquid.

Therefore

$$\frac{D_i}{\delta} A (c_i - c_L) = \frac{k_{eu} E c_L V_L}{K_{mu}} = \theta_u c_L V_L = R_L \quad (9)$$

where  $\theta_u = k_{eu} E / K_{mu}$ . Whence

$$c_L = \frac{D_i A}{\delta} c_i \bigg/ \left( \frac{D_i A}{\delta} + \theta_u V_L \right) \quad (10)$$

and

$$R_L = \theta_u V_L \times \frac{D_i A}{\delta} c_i \bigg/ \left( \frac{D_i A}{\delta} + \theta_u V_L \right) \quad (11)$$

If there were no restriction due to diffusion, the concentration of  $\text{CO}_2$  in the bulk of the liquid would be equal to  $c_i$ , and the true rate  $R$  would be given by

$$R = \theta_u c_i V_L \quad (12)$$

From Equations 10, 11, and 12 it follows that

$$R = \frac{D_i A}{\delta} c_i R_L \bigg/ \left( \frac{D_i A}{\delta} c_i - R_L \right) \quad (13)$$

As the enzyme concentration is raised, the observed rate  $R_L$  tends to a maximum value  $R_m$ , the reaction in the bulk of the liquid becoming so fast that  $c_L$  tends to zero. From Equation 9 it therefore follows that

$$R_m = \frac{D_i A}{\delta} c_i \quad (14)$$

and from Equations 13 and 14 that

$$R = \frac{R_m R_L}{R_m - R_L} \quad (15)$$

$R_m$  can be measured by a direct experiment at high enzyme concentration and Equation 15 then gives a simple way of calculating the true rate  $R$  from the observed rate  $R_L$  when the latter is limited by diffusion. From Equation 14  $D_i A / \delta = R_m / c_i$ ; so that if the treatment is correct the value of  $D_i A / \delta$  obtained in this way should agree with the value obtained from the rate of uptake of  $\text{CO}_2$  in plain physical solution, as described above.

In Fig. 2 on the lower curve is shown the rate of enzymic  $\text{CO}_2$  uptake by 4.2 cc. of M/40 phosphate buffer, pH 7.3, in the presence

of various amounts of carbonic anhydrase at  $0^\circ$ . At low enzyme concentration the points fall on a straight line through the origin, but, as the enzyme is increased, diffusion begins to affect  $R_L$ , which finally tends to a maximum at high concentrations  $= R_m$ . The upper curve of Fig. 2 represents the true rates,  $R$ , as calculated from  $R_L$  and  $R_m$  by Equation 15. They fall, within experimental error, on the straight line passing through the origin

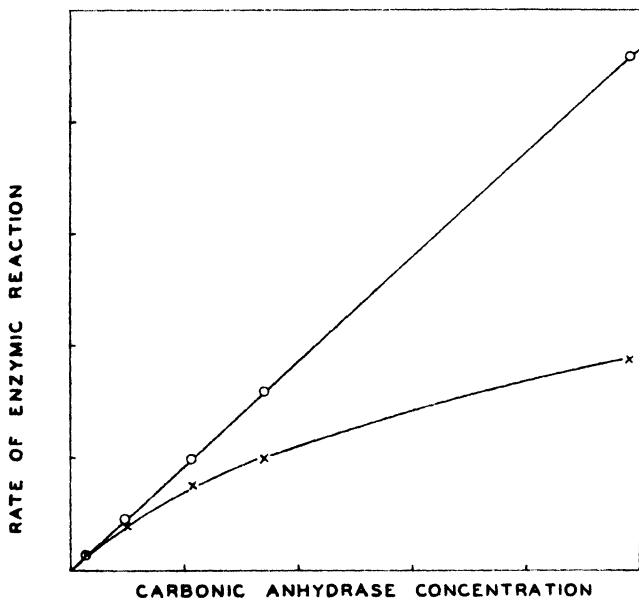


FIG. 2. Rate of  $\text{CO}_2$  uptake by M/40 phosphate, pH 7.3,  $0^\circ$ , in presence of various amounts of carbonic anhydrase.  $\times$  = observed rate;  $\circ$  = rate corrected for diffusion by Equation 15.

and the low values of  $R_L$ . This is to be expected from Equation 8 and is thus a confirmation of the theory.

The value of  $D_i A / \delta$ , given by  $R_m$ , comes out at 0.65. This is about 50 per cent higher than the values given by Fig. 1, for which a less rapid and violent shaker was used than in the present experiment. Two experiments on the rate of  $\text{CO}_2$  uptake in solution under the same shaking conditions as were employed for the readings in Fig. 2 gave values for  $D_i A / \delta$  of 0.64 and 0.63, which agree excellently with the value of 0.65 obtained from  $R_m$ .



*Output Processes*

The general theory has been tested out successfully in four different instances.

*Simple Physical Desolution*—Equation 6 therefore again holds; so that  $\log(p_\infty - p_i)$  plotted against time should accordingly give a straight line. This was checked for the rate of  $\text{CO}_2$  output from a solution of  $\text{CO}_2$  in dilute  $\text{HCl}$ , when the latter is shaken with air at  $\frac{1}{3}$  atmosphere. The value of  $DA/\delta$  came out at 0.44, in exact agreement with the value in Fig. 1, which should indeed be so, as the shaking conditions were identical in the two cases.

*Dehydration of  $\text{H}_2\text{CO}_3$  to  $\text{CO}_2$  in Presence or Absence of Carbonic Anhydrase and at Various pH Values*—Let  $\text{H}_2\text{CO}_3$  concentration in the bulk of the liquid be  $x_L$ .

$K$  = equilibrium constant of the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 = [\text{CO}_2]_\infty / [\text{H}_2\text{CO}_3]_\infty$  = about 900 at  $0^\circ$

$k_0$  = output velocity constant of the reaction  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$  in absence of enzyme

$\theta_0$  = output velocity "constant" for additional rate of dehydration when carbonic anhydrase is added

$k_u$  = uptake velocity constant of the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$  in absence of enzyme

$\theta_u$  = uptake velocity "constant" for additional rate of hydration when carbonic anhydrase is added

Other symbols have the same significance as above.

By the law of mass action

$$K = \frac{k_0}{k_u} = \frac{\theta_0}{\theta_u} = \frac{k_0 + \theta_0}{k_u + \theta_u} \quad (16)^1$$

We then have

$$\begin{aligned} R_L &= \text{observed rate of } \text{CO}_2 \text{ output into gas phase} \\ &= (k_0 + \theta_0)x_L V_L - (k_u + \theta_u)c_L V_L = (k_0 + \theta_0)(x_L - c_L/K)V_L \\ &= \text{at steady state } DA(c_L - c_i)/\delta \end{aligned} \quad (17)$$

$$\begin{aligned} R &= \text{true rate of } \text{CO}_2 \text{ output into gas phase if diffusion were not limiting} \\ &= (k_0 + \theta_0)x_L V_L - (k_u + \theta_u)c_i V_L = (k_0 + \theta_0)(x_L - c_i/K)V_L \end{aligned} \quad (18)$$

$$\begin{aligned} R_m &= \text{maximum rate of } \text{CO}_2 \text{ output into gas phase at very high enzyme concentration} \\ &= DA(x_L K - c_i)/\delta \end{aligned} \quad (19)$$

for in this case the  $\text{CO}_2$  and  $\text{H}_2\text{CO}_3$  are always in equilibrium in the body of the liquid and therefore  $c_L = x_L K$

<sup>1</sup>  $\theta_0$  and  $\theta_u$  are both proportional to the enzyme concentration (but independent of the substrate concentrations if the latter are small compared with the Michaelis constants).

From Equations 17, 18, and 19 it follows that  $R = R_m R_L / (R_m - R_L)$ ; *i.e.*, Equation 15 again holds good. The actual value of  $R_m$ , as shown below, depends upon the pH of the bicarbonate-buffer mixture, whereas in  $\text{CO}_2$  uptake processes  $R_m$  is independent of the pH (between 6.5 and 9.5) of the buffer mixture absorbing the  $\text{CO}_2$ .

Let  $b$  = total  $\text{CO}_2$  in solution in all forms at  $t = 0$

$$= [\text{CO}_2]_L + [\text{H}_2\text{CO}_3]_L + [\text{HCO}_3]_L$$

Under maximum rate conditions

$$c_L = [\text{CO}_2]_L = K[\text{H}_2\text{CO}_3]_L = a_H f[\text{HCO}_3']_L / K_1'$$

where  $a_H$  = hydrogen ion activity of solution

$f$  = activity coefficient of bicarbonate ion

$K_1'$  = apparent first ionization constant of carbonic acid

Therefore

$$c_L = \frac{b}{1 + \frac{1}{K} + \frac{K_1'}{fa_H}} = \frac{b}{1 + \frac{K_1'}{fa_H}}$$

since  $K$  = about 900.

$$\text{At zero time } R_m = \frac{DA}{\delta} c_L = \frac{DA}{\delta} \frac{b}{1 + K_1'/fa_H} \quad (20)$$

The applicability of Equations 15 and 20 has been tested by measurements of the rate of  $\text{CO}_2$  output from mixtures of bicarbonate with cacodylate and acetate buffers at  $0^\circ$ . The  $\text{HCO}_3$  concentration was held constant at 0.0025 M but the pH was varied from 6.3 to 4.7. The results and corrections are given in Table I.

By the law of mass action the true rate of the reaction at  $t = 0$  is

$$\frac{R}{V_L} = k_0(1 + lA')[\text{H}_2\text{CO}_3]_L = \frac{k_0(1 + lA')a_H fb}{a_H f + K_1} \quad (21)$$

where  $A'$  = concentration of buffer anion, *i.e.* cacodylate or acetate

$l$  = catalytic coefficient of buffer anion (see Roughton and Booth (1938))

$K_1$  = true first ionization constant of carbonic acid, assumed  $2.3 \times 10^{-4}$  at  $0^\circ$  (Roughton, unpublished data)

$k_0$  = is assumed to be 2.0 (Roughton, unpublished data) at  $0^\circ$

The last two columns of Table I show that the value of  $R/V_L$  as calculated from Equation 21 agrees to within 1 per cent on the

average with the value of  $R/V_L$  calculated from the observed results with the aid of Equations 15 and 20. The maximum divergence is no more than 10 per cent. The validity of the method for output processes is thus strongly confirmed. In the case of the carbonic anhydrase experiments it is more accurate to obtain  $R_m$  from the rate of  $\text{CO}_2$  output with a high concentration of enzyme added to the bicarbonate-buffer mixture than by Equation 20, as was done in Table I.

*Lag Period in Evolution of  $\text{CO}_2$  from Bicarbonate-Buffer Mixtures*—When bicarbonate and buffer are mixed and shaken together in a manometric vessel, there is, as has long been known, a lag in

TABLE I

*Comparison of Observed Rates (Corrected for Diffusion) and Theoretical Rates of  $\text{CO}_2$  Output from Bicarbonate-Buffer Mixtures of Various pH Values*

$a_H \times 10^5$	$f$	$\frac{R_L}{V_L} \times 10^5$	$\frac{R_m}{V_L} \times 10^5$	$\frac{R_m R_L \times 10^5}{V_L(R_m - R_L)}$	$\frac{k_2(1 + lA)a_H f b \times 10^5}{a_H f + K_1}$
0.57	0.87	1.0	2.3	1.04	1.13
0.98	0.90	1.88	2.7	2.04	2.07
1.95	0.91	3.32	3.05	3.74	3.78
4.8	0.78	6.36	3.28	7.9	8.15
9.6	0.80	11.3	3.39	17.0	16.13
12.0	0.81	13.2	3.42	21.5	20.6
19.1	0.83	17.5	3.46	35.6	33.3

$l$  for cacodylate = 9.0;  $l$  for acetate = 0.6.

the rate of  $\text{CO}_2$  output during the first 15 to 20 seconds compared with that found later (see Fig. 3). Controls show that the lag is not due to slowness of mixing, which should take only 1 second, nor to temperature changes, evolution or absorption of gases other than  $\text{CO}_2$  on mixing, or inertia of the manometric gage fluid. On the stationary film theory, however, the lag is explicable, for  $\text{CO}_2$  cannot diffuse across this film at the full rate until enough  $\text{CO}_2$  has been formed in the body of the liquid by the progress of the  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$  reaction. The actual quantitative effect can indeed be worked out as follows.

If the liquid is well buffered and the pH is  $>6.3$ , the half time of the reaction is 200 seconds or more, so that the  $\text{HCO}_3^-$  and  $\text{H}^+$  concentrations are sensibly constant during the first 20 seconds,

and the rate of the back reaction during this period can be neglected.

The total  $\text{CO}_2$  formed in time  $t$ , if  $0 < t < 20$  seconds, is then equal to

$$V_L k_0 t (1 + lA') [\text{H}_2\text{CO}_3] = V_L k_0 t (1 + lA') a_H f [\text{HCO}_3'] / K_1 = \phi V_L t$$

where  $\phi$  is a constant which can easily be calculated in any given experiment.

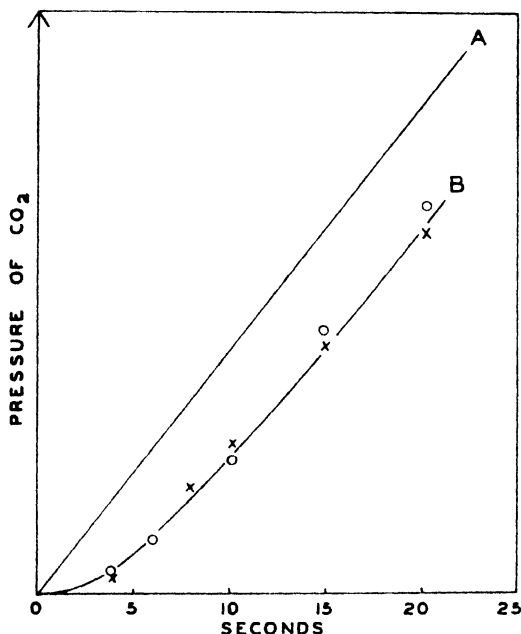


FIG. 3. Effect of diffusion on early stages of  $\text{CO}_2$  output from bicarbonate-cacodylate-enzyme mixture. Curve A, theoretical curve if diffusion is "infinitely" rapid; Curve B, curve obtained by allowing for diffusion in accordance with Equations 25 and 26. O and X = observed rate of  $\text{CO}_2$  output in two experiments.

From Equation 3 the total  $\text{CO}_2$  formed in time  $t = V_g p_i + V_L c_L$ . From Equation 2,  $V_g (dp_i/dt) = DA(c_L - p_i \alpha)/\delta$ .

From these last three equations it follows that

$$\frac{dp_i}{dt} = \frac{DA}{\delta V_g} \phi t - \frac{DA}{\delta} p_i \left( \frac{\alpha}{V_g} + \frac{1}{V_L} \right) \quad (22)$$

Therefore

$$p_i = \frac{\gamma t}{\beta} + \frac{\gamma}{\beta^2} (e^{-\beta t} - 1) \quad (23)$$

where

$$\gamma = \frac{DA}{\delta V_G} \phi, \quad \beta = \frac{DA}{\delta} \left( \frac{\alpha}{V_G} + \frac{1}{V_L} \right) \quad (24)$$

If the diffusion were "infinitely" fast, the pressure of  $\text{CO}_2$ ,  $p'_i$ , which would be developed in time  $t$  would be given by  $V_G p'_i + V_L \alpha p'_i = \phi V_L t$ . Whence

$$p'_i = \frac{\phi V_L t}{V_G + \alpha V_L} \quad (25)$$

From Equations 23, 24, and 25 it follows that

$$\frac{p_i}{p'_i} = 1 + \frac{1}{\beta t} (e^{-\beta t} - 1) \quad (26)$$

$p_i/p'_i$  should therefore be independent of pH, bicarbonate, and enzyme concentration.

With processes which are not too fast,  $\phi$  can be accurately calculated from the manometric readings after 20 seconds, for by then the lag is over: Equation 25 then gives  $p'_i$  and Equation 26 the corresponding value of  $p_i$ . Fig. 3 shows the results of two experiments upon the early stages of  $\text{CO}_2$  output from a mixture containing 0.005 M  $\text{NaHCO}_3$ , 0.006 M cacodylic acid, and 0.006

\* A more elaborate set of equations is necessary if the  $\text{HCO}_3'$  and the  $\text{H}^+$  concentrations do change appreciably during the first 20 seconds, or if the velocity of the back reaction is significant. These equations lead, however, to a final differential equation for  $p_i$  which does not seem to be exactly soluble, though an approximate solution can be obtained in the form of a fairly rapidly convergent power series

$$p_i = \gamma(t^2/2) + \lambda_3 t^3 + \lambda_4 t^4 + \dots \quad (27)$$

where  $\lambda_3, \lambda_4$ , etc., are constants. The justification for assuming such a form is that Equation 23 when expanded gives the convergent power series  $p_i = \gamma(t^2/2) - \gamma(\beta t^3/3) + \gamma(\beta^2 t^4/4) - \dots$ , and when  $t \rightarrow 0$  the values of  $p_i$  given by Equations 23 and 27 should tend to the same limits, but for  $t > 0$  the value of  $p_i$  given by Equation 27 should, from the physicochemical conditions of the problem, always be less than the value of  $p_i$  given by Equation 23.

M Na cacodylate, pH about 6.2, temperature  $0^{\circ}$ , with a small amount of added carbonic anhydrase. Under these conditions  $\beta = 0.17$ , and the lower smooth curve in Fig. 3 is that calculated from Equation 26 by substituting this value of  $\beta$ . The experimental points are seen to fall within experimental error upon the

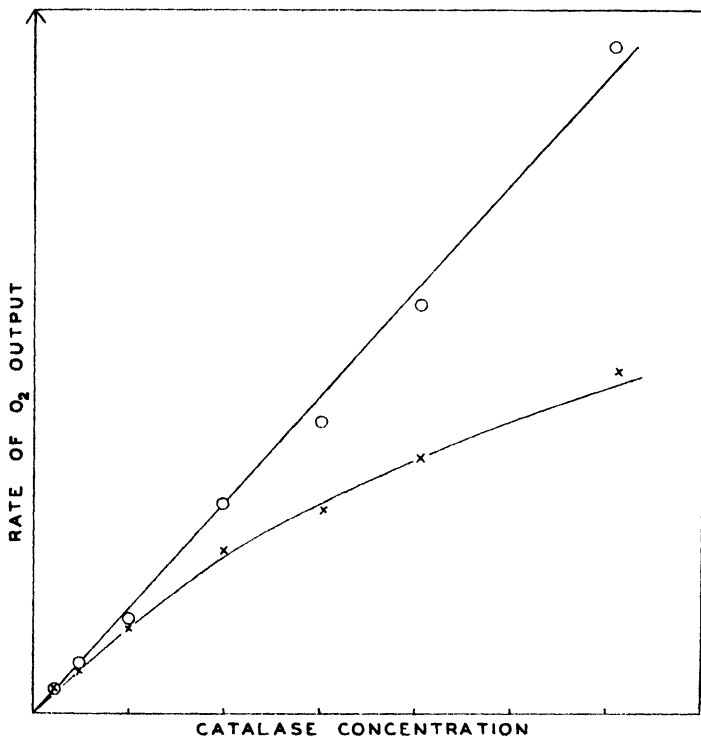


FIG. 4. Rate of  $O_2$  output from 0.0048 M  $H_2O_2$  in M/40 phosphate, pH 7.3,  $0^{\circ}$ , in presence of various amounts of catalase.  $\times$  = observed rates;  $\circ$  = rates corrected for diffusion by Equation 15.

theoretical curve. Equally good agreement was also found for  $CO_2$  output from bicarbonate-phosphate buffer mixtures (pH 6.8) both at  $0^{\circ}$  and at  $15^{\circ}$ .

In the output processes covered by Equations 16 to 26 the  $CO_2$  concentration is generally so much larger than the  $H_2CO_3$  concentration that penetration of the latter into the stationary film has been neglected.

*Decomposition of  $H_2O_2$  by Catalase*—From the kinetics of the  $H_2O_2$ -catalase reaction, it can also be shown that the correction formula (Equation 15) should again be applicable. The points on the lower curve in Fig. 4 give the observed rate of  $O_2$  output  $R_L$  (by the boat-manometric method) from a solution of 0.0048 M  $H_2O_2$  in M/40 phosphate buffer, pH 7.3, at  $0^\circ$ , in the presence of various amounts of purified horse liver catalase (kindly supplied by Professor Keilin and Dr. Mann). The value of  $R_m$  was determined by a special experiment with excess catalase concentration, a further 4-fold increase in the latter producing no change in the rate of  $O_2$  output, thus proving that the maximum rate had been attained. The points on the upper curve in Fig. 4 give the values of  $R$  as calculated from  $R_L$  and  $R_m$  by Equation 15. They fall to within 8 per cent upon the straight line passing through the low values of  $R_L$ , as the theory requires.

The numerical value of  $R_m$  was found to agree quite closely with that for  $CO_2$  at the same concentration, as should indeed be the case, since the diffusion coefficients of  $O_2$  and  $CO_2$  in water agree to within 20 per cent.

It was hoped to include an example of an enzymic  $O_2$  uptake process as well as the  $O_2$  output process just described. Some preliminary experiments were done with catechol oxidase (samples kindly supplied by Professor Keilin and Dr. Mann) but even at the highest available concentrations of the enzyme the speeds of  $O_2$  uptake were not fast enough for the diffusion corrections to be necessary or applicable. In the time at our disposal we were unable to try any other cases.

#### DISCUSSION

In the instances given above, the chemical reactions have been unimolecular with respect to the dissolved gas. With reactions of different order the correction equations need modification. Consider, for example, the general case of  $CO_2$  uptake by buffer solutions in the presence of carbonic anhydrase when the substrate concentration is not assumed necessarily to be small in comparison with the Michaelis constant  $K_{mu}$ . Equation 9 should then read

$$\frac{D_i}{\delta} A(c_i - c_L) = \frac{k_{su} E c_L V_L}{c_L + K_{mu}} = R_L \quad (28)$$

and from Equations 8, 14, and 28 it then follows that

$$R = \frac{R_m R_L}{R_m - R_L} \frac{\frac{R_m - R_L}{R_m} c_i + K_{mu}}{c_i + K_{mu}} = \frac{R_m R_L}{R_m - R_L} \left( 1 - \frac{c_i}{c_i + K_{mu}} \times \frac{R_L}{R_m} \right) \quad (29)$$

In the particular case of  $c_i$  being small compared with  $K_{mu}$  Equation 25 reduces to  $R = R_m R_L / (R_m - R_L)$ ; *i.e.*, Equation 15. Equation 29 must, however, be used when  $c_i$  is of the same order as  $K_{mu}$ .<sup>2</sup> When  $c_i$  is large compared with  $K_{mu}$ , the reaction becomes of zero order with respect to  $\text{CO}_2$  and Equation 29 reduces to  $R = R_L$ ; *i.e.*, no correction for diffusion is required. It is thus clear that before the diffusion correction can be worked out, the chemical kinetics of the particular reaction must be known. This knowledge, however, can often be obtained by working in a restricted range, in which the diffusion corrections are inappreciable; *e.g.*, at very low enzyme concentrations.

Perhaps the most striking result of the present paper is the identity of the initial rate of  $\text{CO}_2$  uptake by plain water with that by phosphate buffer containing a high concentration of carbonic anhydrase. This is a strong confirmation, both of the general theory in this paper and of the whole theory of the stationary film, for such a result would be extremely hard to understand on any other theory. The difficulty of picturing the nature of the physical forces responsible for the maintenance of the films makes such additional evidence certainly welcome.

According to Conant and Shearp (quoted by Davis and Crandall (1930)) the rate of absorption of  $\text{O}_2$  and  $\text{H}_2$  by a saturated solution of oleic acid in water is the same as by water itself in spite of the absorption of the oleic acid at the gas-liquid interface and the increased tendency to foam formation, which would perhaps be expected to alter  $DA/\delta$ . In the instances reported in this paper

<sup>2</sup> Similarly for  $\text{CO}_2$  output from bicarbonate-buffer mixtures it can be shown that if the substrate concentration,  $x_L$ , is not small compared with the Michaelis constant,  $K_{mo}$ , then at  $t = 0$ ,

$$R = \frac{R_m R_L}{R_m - R_L} \left( 1 + \frac{x_L R_L}{K_{mo} R_m} \right) \quad (30)$$



the total protein concentration has always been very low, but if the concentration is raised enough to affect not only the surface tension but also the viscosity of the solution considerable depressions are observed. Thus the rate of solution of gases when shaken with strong hemoglobin solutions or whole blood is found to be only about one-fifth of the rate in pure water. Further work on these lines would be of interest; *e.g.*, on the effect of adding other proteins to strong carbonic anhydrase solutions in regard to the observed values of  $R_m$ .

#### SUMMARY

The chemical kinetics of gas-liquid reactions can only be recorded manometrically (*i.e.* by observations of the change of pressure of the gas phase with time) if the rates so observed are independent of the speed of shaking of the manometric vessel and of the relative volumes of the liquid and gas phases. Otherwise the observed rates depend on the speed of diffusion of dissolved gas between the two phases as well as on the true speeds of the chemical processes.

The effect of diffusion can, however, be allowed for by assuming the existence, at the boundary between the two phases, of a stationary film of liquid, diffusion through which determines the rate of exchange of gas between gas and liquid, the main bulks of which are both assumed to be infinitely well stirred. If the solute molecule with which the gas reacts is of low concentration and diffusivity compared with the dissolved gas, the solute will penetrate into the stationary film so much more slowly than the gas that chemical reaction in the film itself can be neglected. With this simplifying condition it is possible to work out correction equations from which the true rates of chemical reactions can in certain cases be obtained, even when the observed manometric rates are only one-third of the true chemical rates. The range of the manometric method is thus greatly extended.

The equations have been checked by observations on (a) the rate of  $\text{CO}_2$  uptake by, and  $\text{CO}_2$  output from, simple physical solution, (b) the rate of  $\text{CO}_2$  uptake by buffer solutions in the presence of various amounts of added carbonic anhydrase, (c) the rate of output of  $\text{CO}_2$  from bicarbonate-buffer mixtures of various pH values with and without added carbonic anhydrase,

(d) the rate of  $O_2$  output from  $H_2O_2$ -catalase mixtures. Good agreement between theory and experiments is found in each case, and in addition good cross-checks are obtained.

The success so far reached suggests that similar methods may have further biochemical scope and interest.

The experiments in this paper were carried out by Dr. V. H. Booth either alone or with myself. I wish to thank him warmly for this and other help.

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## SUGAR ALCOHOLS

### XXII. METABOLISM AND TOXICITY STUDIES WITH MANNITOL AND SORBITOL IN MAN AND ANIMALS\*

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The fate of mannitol and sorbitol in the animal body has been investigated by a number of workers for many years. Nevertheless, the available data are quite confusing and the older literature relative to the metabolism of these compounds is controversial. This literature has been reviewed by Todd, Myers, and West (1) and by Blatherwick *et al.* (2).

Recently, however, experimental findings of workers in this field have been more consistent concerning certain phases of the metabolism of these sugar alcohols. Mannitol (1, 3, 4) and sorbitol (1, 5) definitely produce hepatic glycogen when fed to fasted rats over a period of days. Glycogen formation also occurs when sorbitol is given intraperitoneally to fasted rats (1, 6), but there is still some controversy concerning glycogen deposition following the oral administration of sorbitol (1, 2, 5). Mannitol does not deposit glycogen in the livers of fasted rats following administration either by stomach tube or intraperitoneally (1, 3).

Carr and Krantz (3) reported that the oral administration of mannitol only slightly increased the respiratory quotient of the rat and produced a mild hyperglycemia in rabbits. Todd *et al.* (1) found that sorbitol elevated the blood sugar in dogs after intravenous injection but mannitol did not. These authors state

\* The expense of this investigation has been defrayed in part by a grant from the Atlas Powder Company, Wilmington, Delaware.

† The material contained in this paper is part of a thesis submitted by Fred W. Ellis to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

further that after intravenous injection of sorbitol into dogs only 40 to 50 per cent of this compound was recovered in the urine. According to Waters (6), intravenous injection of sorbitol into fasting dogs produced only a mild hyperglycemia and markedly depressed the glucose tolerance curve of the normal and depancreatized animal. Smith, Finkelstein, and Smith (7) found that after the intravenous injection of mannitol and sorbitol into normal man 85 and 32 per cent, respectively, of these compounds appeared in the urine.

Since these later reports indicate considerable evidence of sorbitol utilization, this problem has been reinvestigated with particular reference to the possible use of sugar alcohols by man as substitute carbohydrates.

*Materials*—The compounds used in this study were crystalline mannitol and sorbitol and a commercial sorbitol syrup. This syrup is a non-crystallizing, aqueous solution of sorbitol having a polyhydroxylic compound content of about 83 per cent, and is manufactured by the electrolytic reduction of glucose. It is known under the trade name of "arlex."

#### EXPERIMENTAL

*Nutritive Value in Rats*—Groups of white male rats, 20 to 28 days old and weighing about 40 gm., were used in a feeding experiment over a period of 3 months. Twenty animals comprised each experimental group which received in the regular balanced diet 35 per cent dextrose and 5 per cent of one of the sugar alcohols. The diet fed to a control group of animals was composed of 40 per cent carbohydrate consisting entirely of dextrose.

The growth curves of these animals are shown in Fig. 1. These curves do not indicate any significant difference between the relative nutritional values of mannitol and sorbitol under the foregoing experimental conditions. However, dextrose appears to be superior and sorbitol syrup inferior to both mannitol and sorbitol under similar conditions. These results are in accord with those of Ariyama and Takahashi (8) who have previously shown that mannitol is inferior to dextrose in the diet of rats.

*Glycogen Storage in Monkey*—*Macacus rhesus* monkeys were fasted for 24 hours before receiving by stomach tube 8 gm. per kilo of body weight of the respective sugar alcohols. 3 hours later the

animals were anesthetized with sodium pentobarbital and two or three portions of liver were taken from different lobes of each animal for individual glycogen determinations. Small samples of liver were removed under aseptic conditions from two monkeys which recovered from the operation. All of the other animals were sacrificed for pathological examinations.

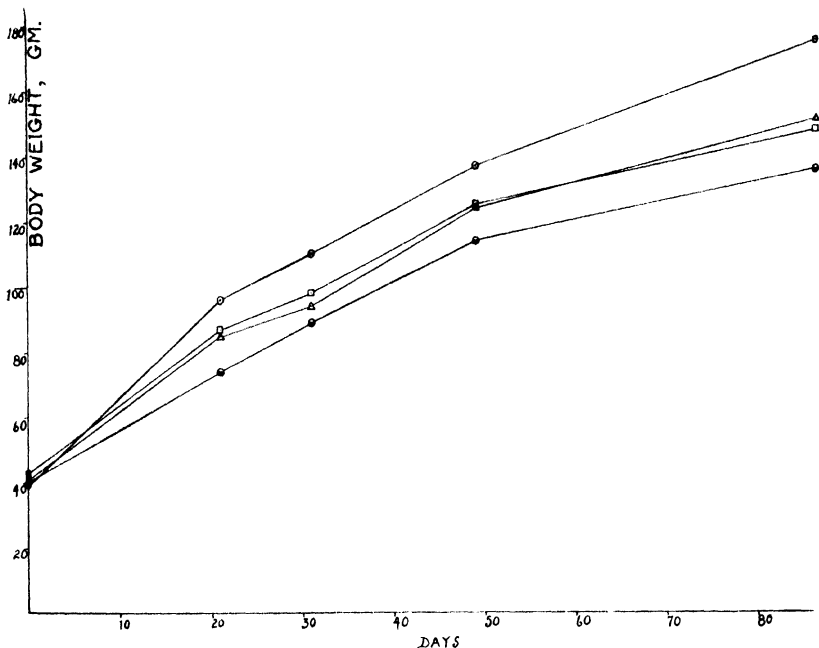


FIG. 1. Growth curves of rats receiving 5 per cent of mannitol and sorbitol in the diet. ○ represents glucose, △ sorbitol, □ mannitol, ● sorbitol syrup.

The glycogen was estimated by the procedure of Good, Kramer, and Somogyi (9) and the dextrose was determined according to the Shaffer-Hartmann method (10). The results are summarized in Table I. These results indicate that sorbitol significantly serves as a precursor of hepatic glycogen, mannitol questionably leads to glycogen deposition, and sorbitol syrup does not give rise to increased glycogen in the livers of rhesus monkeys under these conditions.

*Toxicity in Monkeys*—The chronic toxicity of these compounds was studied in rhesus monkeys by means of two feeding experiments, each of which lasted for 3 months. Nine experimental animals, divided into three equal groups, and two control animals were employed in each experiment. Each such group of animals

TABLE I

*Glycogen Storage in Livers of Rhesus Monkeys after the Administration of Sugar Alcohols by Stomach Tube*

24 hour fasting period.

Control, 30 cc. water			Sorbitol, 8 gm. per kilo		
Monkey No.	Weight	Liver glyco- gen, average	Monkey No.	Weight	Liver glyco- gen, average
	<i>kg.</i>	<i>per cent</i>		<i>kg.</i>	<i>per cent</i>
20	3.79	0.19	12	3.93	0.62
22	3.97	0.06	13	3.48	0.48
96	4.31	0.45	16	3.87	0.32
97	3.73	0.43	95	3.86	0.88
			98	4.18	0.66
			99	3.50	1.35
Mean.....		0.28			0.72
Mannitol, 8 gm. per kilo			Sorbitol syrup, 8 gm. per kilo		
17	3.55	0.62	11	3.51	0.25
89	3.56	0.47	14	3.12	0.22
90	3.98	0.48	15	3.78	0.17
100	3.52	0.49	91	3.88	0.51
101	3.72	0.27	92	4.10	0.45
102	3.38	0.87	94	3.25	0.46
Mean.....		0.53			0.34

was fed a different compound and each monkey received 3 gm. of the designated sugar alcohol in the daily diet.

The blood sugar and urea nitrogen were determined at regular intervals throughout the course of these feeding periods. The values obtained showed no significant variations from the normal values.

At the conclusion of each experiment the animals were sacrificed and routine autopsies were conducted. Sections of the livers and kidneys were prepared and examined by Dr. Henry Wollenweber,

pathologist in this department. Significant degenerative changes which might have been related to toxicity were evident in only one monkey, which was a member of the sorbitol group. In this animal inflammatory changes were noted in the kidney and the cytoplasm of the parenchymal liver cells showed areas of marked rarefaction. We interpret the liver changes as the result of kidney involvement rather than as the result of the feeding of sorbitol, since similar changes were not observed in any of the other animals receiving sorbitol.

*Toxicity in Man*—It has been shown previously (7) that the intravenous administration to normal man of as much as 80 gm. of either mannitol or sorbitol produced no acute toxic symptoms.

In this investigation the chronic toxicity of these compounds was studied in three normal individuals. Each person ingested 10 gm. of mannitol, sorbitol, and sorbitol syrup respectively each day for 1 month in three separate experiments. During each of these periods 24 hour urine samples were analyzed for sugar alcohol content by the method of Todd *et al.* (11). There was no evidence of significant excretion of any of these compounds in the urine.

At the beginning and end of each experiment blood studies were made and the kidney function was estimated. At no time did any appreciable changes occur in the red blood cell count. The renal excretion of phenolsulfonephthalein indicated no kidney damage.

*Laxative Action in Man*—The approximate laxative threshold of each compound was established in twelve normal individuals. The "threshold dose" was considered the minimum amount of each substance which produced very soft or watery stools. This action was produced in the majority of subjects after the oral administration of 10 to 20 gm. of mannitol, 20 to 30 gm. of sorbitol syrup, and about 50 gm. of crystalline sorbitol.

*Respiratory Quotient and Blood Sugar Level in Man*—The observations which comprise this part of the study were made in several normal individuals. Standard procedures employed in basal metabolism and carbohydrate tolerance curve determinations were followed in all experiments. The blood sugar concentration and the respiratory quotient were determined under basal conditions and at periods of  $\frac{1}{2}$  hour, 1 hour, and 2 hours after administration of the compound under investigation.



Expired air of 10 minute periods was collected in a Tissot tank and samples were taken for determination of respiratory quotient by the Haldane-Henderson technique. Capillary blood for sugar determinations was obtained from the finger tip after each respiration period and was analyzed by the Folin method (12).

Each basal subject ingested a definite quantity of dextrose and remained in an inclined position throughout the experiment. Under similar conditions on 3 subsequent days each person received an equal quantity of mannitol, sorbitol, or sorbitol syrup. In this routine manner it was possible to study the metabolic activity of each of these compounds in each individual.

TABLE II

*Respiratory Quotient and Blood Sugar Level in Man after Dextrose, Sorbitol, and Mannitol*

Substance	Amount administered	No. of experiments	Average values							
			Basal		$\frac{1}{2}$ hr.		1 hr.		2 hrs.	
			R.Q.	Blood sugar	R.Q.	Blood sugar	R.Q.	Blood sugar	R.Q.	Blood sugar
	gm.			mg. per cent		mg. per cent		mg. per cent		mg. per cent
Dextrose.....	50	2	0.81	82	0.79	123	0.87	115	0.92	109
Sorbitol.....	50	2	0.78	93	0.83	104	0.93	97	0.98	93
Dextrose.....	25	7	0.77	107	0.77	147	0.82	126	0.83	105
Sorbitol.....	25	7	0.77	104	0.81	110	0.80	105	0.83	103
" syrup....	25	5	0.77	98	0.77	100	0.79	98	0.79	96
Mannitol.....	25	5	0.78	96	0.77	95	0.79	94	0.79	94

In most of these tests only 25 gm. of the respective compound were ingested, since larger amounts usually produced catharsis, which interferes to some extent with quantitative studies. In two subjects 50 gm. of sorbitol were used without severe intestinal disturbance.

The average values obtained in these experiments are shown in Table II. These results demonstrate the capacity of sorbitol to increase the respiratory quotient without appreciably elevating the blood sugar. Mannitol and sorbitol syrup did not significantly influence either the blood sugar or the respiratory quotient.

## SUMMARY

1. In mice and rats the laxative action of mannitol and sorbitol limits nutritional studies when these compounds comprise a portion of the diet.

2. In the rhesus monkey, sorbitol is capable of storage as glycogen in the liver of the fasted animal. Under the same conditions mannitol is incapable of producing significant glycogen storage.

3. The feeding of 3 gm. per day of mannitol and sorbitol to rhesus monkeys over a period of 3 months produced no histopathological findings or toxicological indications which were attributed to these compounds.

4. In man, the daily ingestion of 10 gm. of mannitol and sorbitol, respectively, for 1 month produced no significant changes in the non-protein nitrogen, CO<sub>2</sub>-combining power of the blood, or the red blood cell count. The phenolsulfonephthalein test indicated no kidney damage.

5. The approximate threshold of laxative action in man was determined.

6. In normal human subjects, sorbitol increased the respiratory quotient above the basal level. The effect was similar within 2 hours to that of an equal quantity of dextrose. The blood sugar curve remained practically normal after sorbitol. Mannitol and sorbitol syrup did not significantly influence either the blood sugar or respiratory quotient.

7. The laxative effect of mannitol and sorbitol syrup is greater than that of crystalline sorbitol.

The authors wish to thank Dr. C. Jelleff Carr and Dr. W. E. Evans, Jr., for their assistance in these studies.

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# THE ISOLATION OF BACTERICIDAL SUBSTANCES FROM CULTURES OF *BACILLUS BREVIS*

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Two bactericidal substances, gramicidin and tyrocidine hydrochloride, have been prepared in crystalline form from cultures of an aerobic sporulating bacillus (1, 2). This communication will describe in greater detail the isolation and characterization of these substances.

The bactericidal material was first prepared from a strain of organisms (BG strain) isolated from soil and later identified as *Bacillus brevis*. Other strains tested since that time have yielded material with similar activity from which in some cases crystalline gramicidin and tyrocidine hydrochloride have been isolated and identified (3). In addition, McDonald (4) has reported the preparation by Hoogerheide of bactericidal material of similar properties from various aerobic sporulating organisms, and the isolation from one strain of a substance which was recognized as possibly identical with gramicidin. What follows will show that the identity is indeed complete.

Previous work had shown that acidification of the autolyzed culture gives a precipitate from which the bactericidal material could be recovered in two different forms, (Preparation A) a water-soluble form obtained by extraction with neutral buffer (5), and (Preparation B) an alcohol-soluble, water-insoluble product (6) later designated tyrothricin, by extraction with alcohol or acetone without neutralization. Tyrothricin (Preparation B) is the source from which the two crystalline substances were obtained. The water-soluble material (Preparation A) is protein in nature and on treatment with acid alcohol or acetone yields an inactive precipitate and an active soluble fraction apparently identical

with tyrothricin. Since tyrothricin cannot again be brought into solution in aqueous buffer, it is believed to be a product of some form of alteration or degradation of the soluble protein material. Chemical studies show that gramicidin and tyrocidine are polypeptides resistant to common proteolytic enzymes (1, 7). When the water-soluble fraction is treated with proteolytic enzymes, water-insoluble matter with the full antibacterial activity of the original solution is precipitated as the enzymatic action proceeds. The above considerations suggest that gramicidin and tyrocidine exist in the culture autolysate preformed and combined in some unknown fashion in a protein or proteins, the bactericidal activity of which is due in part at least to these component polypeptides.

All processes for the fractionation of tyrothricin described in the literature depend upon the fact that the salt, tyrocidine hydrochloride, is considerably less soluble than the neutral gramicidin in mixtures containing ether. The original method of fractionation (1) called for the precipitation of alcohol solutions by ether, followed by a step in which acetone-ether mixtures were employed. In a later simplification of the first procedure (2) the acetone and ether alone were used. These separations indicated a content of approximately 10 to 20 per cent gramicidin and 40 to 60 per cent tyrocidine hydrochloride in tyrothricin. Other substances are probably present but the biological and chemical properties of tyrothricin which have been observed are not different from those to be expected from a mixture of the two crystalline substances.

Because of the difficulty and expense of obtaining the maximal yield of crystalline gramicidin and tyrocidine, the cruder tyrothricin has often been used in studies of the antibacterial effect of the material *in vivo*. For such purposes the product may be extracted with ether once or twice to remove fats and waxes and may require reprecipitation from alcohol solution with saline. When the mechanism of the biological effects is under study, it is essential to work with the crystallized substances, since the two components so far isolated differ markedly in most of their properties.

Some of the biological properties of gramicidin and tyrocidine have been reported (1-6, 8-10). A brief summary of the principal ones should probably be given here. Both substances are markedly bactericidal for Gram-positive microorganisms; grami-

cidin is effective in amounts as low as 1  $\gamma$  per billion organisms, and tyrocidine in quantities of 25 to 50 times this amount. The latter substance, in the absence of inhibitors like peptone media, is also effective against Gram-negative bacteria; furthermore, its killing effect is accompanied by bacteriolysis whenever the organisms being tested possess an active autolytic system. Both substances affect metabolic processes of bacteria, tyrocidine blocking all of the oxidative systems studied, gramicidin on the other hand appearing to affect only certain individual reactions. Both compounds can exert a protective antibacterial action in mice infected intraperitoneally with susceptible microorganisms, but gramicidin protects the animals at a level (around 1  $\gamma$  for  $10^4$  fatal infective units of pneumococcus Type I) one-fiftieth as high as that required for tyrocidine. Both substances are hemolytic—the hemolysis by gramicidin, however, though caused by much smaller amounts, is slow and is entirely prevented by the presence of low concentrations of glucose or mannitol. Both compounds are highly toxic to animals when injected into the blood stream; they appear, however, to exhibit little toxicity when applied locally, as, *e.g.*, by the subcutaneous, intramuscular, or intrapleural route. The substances are both rich in nitrogen and give color reactions for amino acids. Chemical data will be presented in an accompanying communication.

#### EXPERIMENTAL

*Preparation of Tyrothricin*—Tyrothricin is the name recently given to the alcohol-soluble fraction of the acid precipitate from a culture medium. The earlier method of preparation (6) is followed with some modifications (3). Extraction with ether once or twice removes fatty acids and waxy impurities; extraction with dioxane has not generally been used. A crystalline, optically inactive fatty acid, tentatively identified as stearic acid by solubility and analysis, makes up a large portion of the ether extract.

So prepared, the material contains about 12 to 13 per cent of nitrogen. It gives positive biuret, xanthoproteic, Millon, and Hopkins-Cole reactions and a negative Sakaguchi reaction. That these tests were all reported as negative when the material was originally described (6) is undoubtedly due to the insolubility of tyrothricin in the presence of electrolytes. Unless dissolved first

in alcohol or acetic acid, the material reacts with the test reagents only with the greatest difficulty.

*Preparation of Gramicidin*—Tyrothricin is extracted repeatedly with a mixture of equal volumes of acetone and ether by mechanical shaking in a vessel containing glass beads. Two or three extractions with 10 to 20 parts by weight of the solvent will remove most of the gramicidin. Evaporation of the extracts gives a brownish gummy residue which is taken up in about 5 times its weight of warm acetone. A better guide to the appropriate amount of solvent is the viscosity of the solution, which should be brought to the point where the mixture is no longer syrupy but flows freely. In such a solution crystallization is spontaneous and complete within a few hours. Recrystallization from the minimum amount of boiling acetone (or dioxane) two or three times, with removal of insoluble matter and washing of the crystals with cold acetone and with ether, gives an essentially pure product. Crystalline gramicidin is far less soluble in acetone than the amorphous gummy residue from which it is obtained. The typical crystals are tiny platelets with pointed ends, the outline being like that of a biconvex lens. When slowly deposited the platelets develop rectangular ends.

Purified by repeated recrystallization until its properties are constant, gramicidin is a colorless substance. The melting point is quite sharp at 230–231° (capillary tube in a copper block, corrected), or 228–230° (hot stage, uncorrected). The optical rotation in alcohol solution is low.

$$[\alpha]_D^{25} = +5^{\circ} \text{ (} c = 0.4\% \text{ in 95\% alcohol)}$$

$$[\alpha]_D^{25} = +2.5^{\circ} \text{ (} c = 1.5\% \text{ in absolute alcohol)}$$

Gramicidin is soluble in the lower alcohols, acetic acid, and pyridine; moderately soluble in dry acetone and dioxane; and almost insoluble in water, ether, hydrocarbons, and chlorinated hydrocarbons. When a solution of 20 to 50 mg. per cc. in alcohol is diluted to 1 mg. per cc. with distilled water or glucose solutions, an opalescent solution is formed but there should be no flocculation. On dilution with electrolyte solutions there is immediate flocculation.

Elementary analysis reveals carbon, hydrogen, and nitrogen but no sulfur, halogen, or phosphorus. The composition is (aver-

age values) C 62.7, H 7.59, N (Kjeldahl) 14.8.<sup>1</sup> The lower nitrogen value reported before was obtained by micro-Dumas combustion. It has become increasingly evident that Dumas combustions with gramicidin were unreliable under the conditions employed. With thoroughly dried identical samples, the nitrogen values fluctuated in all from 13.3 to 14.7 per cent, usually coming near the middle of the range. Carbon and hydrogen analyses on the same preparations were entirely satisfactory. Consistent nitrogen determinations have been accomplished by means of a micro-Kjeldahl method in which pretreatment with hydriodic acid is employed (11). This procedure, followed by a 2 hour digestion with selenium-copper catalyst, was chosen, since ordinary Kjeldahl analyses revealed only 88 to 95 per cent of the total nitrogen of pure tryptophane. With the modified method 99 to 100 per cent is recovered, and gramicidin, which is rich in tryptophane, gives consistently the value presented above.

It will be noted that the nitrogen content of gramicidin has been brought into agreement with that of Hoogerheide's Fraction II (4). The only other obstacle to the complete identification of Hoogerheide's substance with gramicidin was an earlier statement that the crude material did not give protein color reactions. This statement has been corrected above. All other details of physical and chemical properties and crystal form are in complete agreement. Therefore, it appears certain that Hoogerheide, by a purification process which is a rearrangement of the same steps we have described, has prepared gramicidin itself rather than a new substance with similar activity.

Fig. 1 shows an ultraviolet absorption curve of gramicidin, obtained through the kindness of Dr. G. I. Lavin. The curve was prepared from data determined with a Spekker spectrophotometer and a small Hilger quartz spectrograph. Gramicidin was photographed in alcohol solution. A very similar curve was obtained by Hoogerheide with his Fraction II (4). The curve in Fig. 1 is presented here because it shows greater detail in the region around 2750 Å. The absorption curve of tyrocidine hydrochloride is of the same general type.

<sup>1</sup> The authors wish to thank Mr. A. Elek for carrying out the carbon and hydrogen analyses, as well as numerous Dumas determinations in attempts to find appropriate conditions for combustion.



The maximum content of gramicidin in tyrothricin is probably around 20 per cent and only about one-half to two-thirds this amount can conveniently be obtained in crystalline form. In working up accumulated residues, frequent use has been made of precipitation from alcohol solutions by 10 to 15 volumes of ether. Gramicidin remains largely in the solution and is recovered after evaporation.



FIG. 1. Absorption curve of gramicidin.  $d$  = optical density = logarithm of incident over transmitted light intensity. Gramicidin solution, 0.11 mg. per cc. in alcohol; cell length, 1 cm.

*Preparation of Tyrocidine Hydrochloride*—The portion of tyrothricin (around 85 per cent) which remains insoluble when gramicidin is extracted in acetone-ether is dissolved in about 4 times its weight of boiling absolute alcohol. The solution is cooled and there is added alcoholic hydrogen chloride equivalent to 0.1 mole per liter. Microscopic clusters of needles are deposited which are separated and recrystallized from absolute methanol. The solubility is decreased by addition of alcoholic hydrogen chloride; to cut down any possible alcoholysis, the acid is added after the solution has cooled. Tyrocidine hydrochloride crystallizes from methanol as fine colorless needles or rods (truncated at an angle of approximately  $52^\circ \pm 5^\circ$ , showing parallel extinction) melting

unsharply and with decomposition at about 240° (corrected; capillary in a copper block). The optical rotation is high.

$$[\alpha]_D^{25} = -101^{\circ} \text{ (} c = 1\% \text{ in 95\% alcohol)}$$

After about three recrystallizations the analysis is constant and gives C 59.6, H 6.66, N (Kjeldahl) 14.31, Cl 2.7. Material with a lower carbon content is removed during purification. The nitrogen analysis given represents an improvement over the low and variable results of the Dumas combustion (see above). Tyrocidine hydrochloride is identical with the substance first called *graminic acid*, although the description of properties in the preliminary announcement had to be revised when larger quantities of material became available for study. *Gramidinic acid* on the other hand was never completely characterized, and is now known to have been essentially a crystalline mixture of tyrocidine and its hydrochloride.

Tyrocidine hydrochloride is moderately soluble in methyl and ethyl alcohols, acetic acid, and pyridine (the solubility being increased by a small percentage of water), sparingly soluble in water, acetone, and dioxane, and insoluble in ether and hydrocarbon solvents. An alcohol solution can be diluted to give a clear aqueous solution containing 5 to 10 mg. per cc. but electrolytes immediately precipitate the material. A solution in distilled water containing 1 mg. per cc. or even less has an obviously low surface tension and behaves like a soap or detergent solution. Like some of the cationic detergents (12), it precipitates a number of soluble proteins. Gramicidin solutions or suspensions do not precipitate proteins.

#### SUMMARY

From peptone cultures of *Bacillus brevis* there have been isolated two crystalline compounds, designated gramicidin and tyrocidine hydrochloride, which are antagonistic toward a variety of microorganisms. The preparation and characterization of these substances are described.

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## THE OCCURRENCE OF *d*-AMINO ACIDS IN GRAMICIDIN AND TYROCIDINE

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Amino acids having a dextro configuration have been found in natural material from two sources.<sup>1</sup> Jacobs and Craig obtained *d*-proline from ergotinine (4) and later other ergot alkaloids, and Ivánovics and Bruckner found *d*(-)-glutamic acid as the principal constituent of the P-antigen of *Bacillus anthracis* and other microorganisms of the related *Bacillus mesentericus-subtilis* group (5). Inasmuch as gramicidin and tyrocidine were obtained from a related aerobic spore-forming organism, *Bacillus brevis* (6-8), it was thought to be of interest to investigate the configuration of the amino acids present in these two polypeptides.

The enzymatic method (9) which was used made it possible to discover the presence of *d*-amino acids at a time when the quantities of pure material available were too small to permit the isolation and polarimetric identification of individual amino acids. This method depends upon the measurement of oxygen uptake and ammonia production when the specific *d*-amino acid oxidase of Krebs (10) acts upon a hydrolysate. Mention was made earlier of the finding of *d*-amino acids in gramicidin and tyrocidine (6);

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<sup>1</sup> Kögl and Erxleben (1) have described the preparation from tumor proteins of amino acids which, judged on a basis of optical rotation, had small proportions of the *d* forms present. Later investigations by a number of workers have shown that the findings were probably explained by racemization and fractionation during isolation of the amino acids rather than by the occurrence of the *d* forms in the original material (see *e.g.* Dunn (2) and Schoenheimer and Ratner (3)).

the data are presented here, and an accompanying communication reports the actual isolation of *d*-leucine from gramicidin (8).

The possibility existed that the observed oxidation might have been brought about by some enzyme, other than *d*-amino acid oxidase, also present in the more or less crude enzyme preparation employed. However, there are a number of indications that this cannot be the case. Another communication shows that about 92 per cent of the total nitrogen of hydrolysates of both gramicidin and tyrocidine is present in typical  $\alpha$ -amino acids and ammonia (8). Since the ammonia production by the oxidase was practically equivalent to the oxygen uptake, and corresponded to a considerable proportion of the total nitrogen, it was necessary to conclude that it was actually the result of the oxidation of *d*-amino acids. This conclusion has been made still more convincing through the removal, according to the procedure of Negelein and Brömel (11) of the major part of the flavin-adenine prosthetic group from the enzyme protein. When the pure flavin-adenine dinucleotide of Warburg and Christian (12) was added back to the protein component, there was a 3-fold increase in the rate of oxidation of gramicidin hydrolysate. This finding is additional proof that the oxidizing enzyme actually was *d*-amino acid oxidase.

#### EXPERIMENTAL

*Preparation of Hydrolysates*—Gramicidin and tyrocidine hydrochloride were hydrolyzed in hydrochloric acid containing acetic acid. A carbon dioxide atmosphere was provided during the hydrolysis and until the strong acid had been removed by evaporation. These conditions were such that only a negligible amount of tryptophane was destroyed or racemized (*cf.* Experiment 4 in Table I). Further details concerning the hydrolysates will be found in another place (8).

*Enzyme Preparation*—A detailed description of the enzymatic method will be given in a separate publication by one of us (L.). In most of the experiments described here a dry preparation was used, obtained by acetone precipitation of an extract of acetone-dried lamb kidney. Of this dry powder a 10 per cent solution was prepared in 0.2 M pyrophosphate of pH 8.3 (12), containing one-fourth its volume of gum ghatti solution (2 gm. of gum ghatti extracted with 100 cc. of hot water). With *d*-alanine, 0.25 cc. of

the concentrated enzyme solution absorbed 40 to 70 c.mm. of  $O_2$  in 10 minutes.

*Oxidase Experiments*—Ordinary respiration vessels of 8 cc. total volume were used, with 0.5 to 1.0 cc. of hydrolysate (neutralized to phenolphthalein) in the main compartment. 0.25 cc. of enzyme solution was placed in the side arm. A small crystal of thymol was added to each vessel to prevent bacterial growth. The ex-

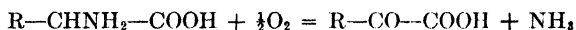
TABLE I  
*Oxidation of Gramicidin Hydrolysates by d-Amino Acid Oxidase*

Experiment No.	Hydrolysate	Total $\alpha$ -NH <sub>2</sub> -N	Time of oxidation	d-Amino N found			Remarks
				From O <sub>2</sub> uptake	From NH <sub>3</sub> produced	From NH <sub>3</sub> , per cent of $\alpha$ -NH <sub>2</sub> -N	
		mg.	hrs.	mg.	mg.		
1	HA2	0.978	17	0.52	0.43	44	Enzyme I
				0.52	0.42	43	
2	HA4. Hydrolysis 80% complete	0.85	23	0.35	0.30	35	
2a	" "	0.85	4	0.32	0.29	34	" II
							Reconstituted enzyme
2b	HA4. Tryptophane-free fraction	0.626	4	0.32			" "
2c	HA4. Tryptophane fraction	0.419	4	0			" "
3	HA5	0.480	19	0.32	0.22	46	Enzyme III
3a	" Tryptophane-free fraction	0.309	19	0.27	0.21	68	" III
4	"Hydrolyzed" tryptophane	0.20	23	0			" II

periments were carried out at 37° with air as the gas phase and with sodium hydroxide in the center cup. After equilibration the enzyme was added from the side arm and shaking was continued for 17 to 20 hours to obtain as complete a reaction as possible. The largest part of the oxygen uptake, however, occurred in the first 3 hours (*cf.* Experiments 2 and 2a in Table I) indicating the presence of fairly rapidly reacting *d*-amino acids.

At the end of the experiment the sodium hydroxide was removed and 2.5 or 3.0 cc. of 5 per cent trichloroacetic acid were added to the main compartment of the vessel. The contents were then transferred to a small centrifuge tube. After centrifugation, ammonia was determined in an aliquot sample. The ammonia was driven over either *in vacuo* after the addition of borate (Parnas (13)) or by steam distillation from calcium hydroxide, and finally determined by titration. Each experiment was accompanied by a blank determination of the oxygen and ammonia for the enzyme alone. These are deducted in the figures given in Table I. The oxygen taken up by the enzyme blank was not greater than 10 to 20 c.mm.

For the calculation of the percentage of *d*-amino acid, given in next to the last column of Table I, the more reliable figures from the ammonia production were used. Compared with the ammonia values, the oxygen consumption was usually from 15 to 30 per cent above the values to be expected from the equation,



In a recent paper on *d*-amino acid oxidase, Klein and Handler (14) reported similar discrepancies with a number of amino acids.

Similar experiments were performed with the reconstituted enzyme. The removal of the prosthetic group was carried out by the procedure described by Negelein and Brömel (11). Without the use of a cooled centrifuge it was not feasible to remove completely the flavin-adenine. On addition of a pure preparation of flavin-adenine dinucleotide<sup>2</sup> the rate of oxidation of *d*-alanine by the protein component rose 3-fold. Experiments with gramicidin hydrolysate and the tryptophane-free fraction of it, in which the protein was employed with and without the added flavin component, gave a similar result. Fig. 1 shows the initial rates of oxidation in one of the experiments.

The initial rates found with both crude and purified enzyme preparations are always higher than would be expected if *d*-leucine alone were present. There is thus an indication of the presence of another and faster reacting *d*-amino acid. The tryptophane fraction contained no detectable *d*-amino acid (Table I) and, in

<sup>2</sup> A sample of pure barium flavin-adenine dinucleotidate has been kindly supplied to the senior author by Professor Otto Warburg.

confirmation of this finding, crystalline *l*-tryptophane was isolated (8).

*Experiments with Tyrocidine*—Oxidase experiments showed appreciable quantities of *d*-amino acids in tyrocidine hydrolysates. Ammonia production indicated that somewhat more than 20 per cent of the  $\alpha$ -amino acids were of the *d* configuration. This might be considered as a minimum value, since with tyrocidine the ammonia determinations indicated only about 50 per cent of what would have corresponded to the oxygen consumed. In addition, the analysis of ammonia produced required the correction for a

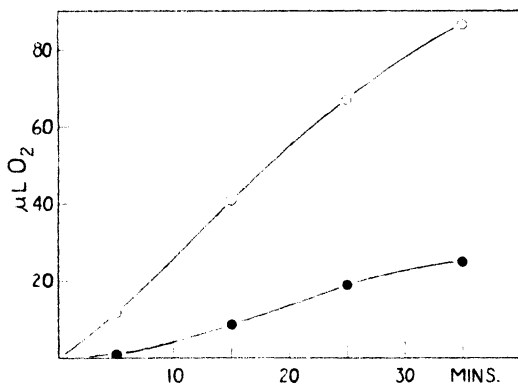


FIG. 1. Initial oxygen uptake by acid hydrolysate of gramicidin and *d*-amino acid oxidase protein. ● without added flavin-adenine dinucleotide; ○ with added flavin-adenine dinucleotide.

rather large amount of preformed ammonia in the tyrocidine hydrolysate. A dicarboxylicamino acid fraction prepared from tyrocidine showed very little, if any, *d*-amino acid.

#### DISCUSSION

The *d*-leucine and *l*-tryptophane recovered from gramicidin and the 68 per cent of *d*-amino acids found in the tryptophane-free fraction are sufficient evidence that the amino acids of gramicidin are not present as racemates. The *d*-leucine  $\beta$ -naphthalenesulfonate isolated corresponds to about one-half the total of 45 per cent determined as *d*-amino acids by the oxidase method. Since a large part of the remaining amino acid is alanine (8), there is a



considerable possibility that all or part of it could be present as *d*-alanine. The rather high rate of oxidation points in this direction.

Gramicidin, then, presents a picture of a polypeptide composed of amino acids, some of which occur in the *d* form and others of which occur in the *l* form. This interesting type of structure, which was also indicated in the polypeptide component of ergotinine (4), may well be responsible for the resistance of gramicidin (and tyrocidine) to ordinary proteolytic enzymes. Possession of a structure of this sort may conceivably be important in connection with the known antibacterial and toxic properties of these polypeptides, either by contributing a toxicity in itself, or by making difficult the destruction and removal of a molecule toxic for other reasons.

The findings of Ivánovics and Bruckner (5) and the above results furnish two cases of the occurrence of *d*-amino acids in the aerobic sporulating bacteria. Berger, Johnson, and Baumann have described *d*-peptidase activity in a number of microorganisms (15), one of which, *Bacillus megatherium*, belongs to the same general group. Inasmuch as the organisms of this group are wide-spread in nature, and are found for example in certain cheeses, it seems possible that the animal organism is, on occasion, confronted with the task of disposing of *d*-amino acids liberated in the intestinal tract. If this should be the case, it would provide one use for the oxidase, occurring in animal tissues, specifically capable of breaking down *d*-amino acids.

#### SUMMARY

Enzymatic assay with *d*-amino acid oxidase indicates that 45 per cent of the  $\alpha$ -amino acids of gramicidin hydrolysates have the *d* configuration. Tyrocidine appears to contain *d*-amino acids amounting to 20 per cent of its  $\alpha$ -amino acids.

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# THE CHEMICAL NATURE OF GRAMICIDIN AND TYROCIDINE

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The work reported here is a continuation of earlier investigations on the chemical nature of two bactericidal substances, gramicidin and tyrocidine, isolated from cultures of *Bacillus brevis* (1-3). Christensen, Edwards, and Piersma are at the same time submitting a report of work which has been carried on independently in their laboratory (4).

Tyrocidine hydrochloride and gramicidin are both colorless crystalline substances, soluble in organic solvents and optically active. Their biological properties offer certain interesting contrasts (1, 5). Gramicidin appears to be more specific in its antibacterial action and to retain this property to a greater extent in the presence of animal tissue. Accordingly, the study of its chemical nature has been pursued more actively than that of tyrocidine.

## *Gramicidin*

Gramicidin is found to be a polypeptide containing 14.8 per cent of nitrogen, and no free amino or carboxyl groups. It appears to be completely resistant to trypsin, pepsin, and papain, but is hydrolyzed by acids. Of the total nitrogen of the acid hydrolysate, 37.3 per cent is found in tryptophane, 53.9 per cent in other  $\alpha$ -amino acids, 7.8 per cent as an additional primary amino compound, and 1.4 per cent as ammonia. The total nitrogen (100.4 per cent) can therefore be accounted for.

Of the tryptophane, 40 per cent was isolated as crystalline *l*-tryptophane. Earlier experiments with Dubos and Lipmann (1, 6) had shown by the use of Krebs' *d*-amino acid oxidase that

about 45 per cent of the  $\alpha$ -amino acids were of the "unnatural" *d* configuration. This finding has been qualitatively confirmed by the actual isolation of optically and analytically pure *d*-leucine, through the  $\beta$ -naphthalenesulfonate (7). The amount of the derivative isolated accounted for 16.1 per cent of the total nitrogen of gramicidin, and one-half of this was recovered as the free amino acid. Correction for solubility or a quantitative determination has not yet been attempted. Alanine was detected in an amount equivalent to 25 to 35 per cent of the total nitrogen but the method of Kendall and Friedemann (8) which was used is not sufficiently specific to enable the alanine content to be ascertained on this basis alone.

The other amino component, referred to above, which comprises 7.8 per cent of the total nitrogen, reacts with nitrous acid and is titrated as a base in acetone solution, but does not react as an  $\alpha$ -amino acid in the Van Slyke-Dillon procedure (9). Sodium periodate, under the conditions employed for amino acids by Van Slyke *et al.* (10), decreases the amino nitrogen content of gramicidin hydrolysate without affecting the  $\alpha$ -amino acid content. Therefore, the substance destroyed by periodate is this same non- $\alpha$ -amino component. The products of periodate action are formaldehyde, ammonia, and a substance which appears to be an aldehyde or keto acid. We know from the work of Nicolet and Shinn (11) that periodate attacks 1,2-aminoalcohol compounds in much the same way as it does 1,2-glycols (12), producing two carbonyl compounds and a mole of ammonia. The observations therefore indicate that gramicidin hydrolysates contain an aminoalcohol compound (or compounds) having adjacent amino and hydroxy groups, one of which is on the end of a carbon chain. The substance cannot be serine, threonine, or hydroxyglutamic acid since it is not a  $\beta$ -hydroxy- $\alpha$ -amino acid, and not hydroxyllysine since basic amino acids are absent. It does not seem to be isoserine, but may be a higher aliphatic aminoalcohol acid. In any case it must be the principal unique component of gramicidin aside from the *d*-amino acids.

The data so far given are independent of considerations of molecular weight. Before chemical data can be used in the assignment of a precise empirical formula, it is first necessary to ascertain the order of magnitude of the molecular weight by physical meth-

ods. With the rather complex gramicidin the difficulty of obtaining precise physical or chemical data is sufficiently great so that as yet it is possible only to limit the choice to a few alternative formulas.

The lowering of the freezing point of camphor by gramicidin indicates molecular weights in the range of 1250 to 1550, but the variability of the small depressions observed makes it difficult to fix this quantity more accurately. The possibility exists that the cryoscopic camphor method may be inapplicable to gramicidin but there is no evidence that this is the case. Precautions were taken to avoid decomposition by heating. A preliminary attempt was made to arrive at the molecular weight by isothermal distillation of alcohol solutions, but equilibration of the vapor pressures was very slow and critically subject to local variations in temperature.

Another approach to the molecular weight depends upon the fact that all chemical constituents must be present in integral molecular quantities. Two components, tryptophane residues and hydroxyl groups, are present in amounts suitable for the estimation of minimum molecular weights. Tryptophane analyses of hydrolysates indicate a molecular weight in the series 510, 1020, 1530, etc. Estimations of the hydroxyl group of unhydrolyzed gramicidin by quantitative acetylation indicate one hydroxyl group per  $1220 \pm 80$  of molecular weight.

If considerable margin is allowed for error, the results given above leave in the main three possibilities for the molecular weight: (a) one in the range of 1000 to 1150, (b) one of 1400 to 1600, or (c), if the cryoscopic determination is rejected, a higher multiple of 510 such as  $2 \times (a)$ ,  $(a) + (b)$ , etc.

Accordingly, in Table I the analytical data are recorded in terms of two units, of 11 and 15 nitrogen atoms. Empirical formulas which correspond to these units are  $C_{55}H_{79}N_{11}O_{10}$  (mol. wt. 1054) and  $C_{74}H_{106}N_{15}O_{13}$  (mol. wt. 1413). These are offered as satisfactorily expressing the analytical data which have been obtained so far. It is obvious that a change of 1 carbon atom or 2 atoms of hydrogen in such large molecules would barely be detectable in the elementary analyses, and either of the formulas could be in error by such an amount or by the difference  $C_6H_7NO$ .

From the data in Table I it is apparent that the neutral

gramicidin liberates on hydrolysis roughly equal amounts of basic (amino) and acidic groups. It will be seen that the aminohydroxy nitrogen is appreciably less than the non- $\alpha$ -amino nitrogen but these quantities are both differences of larger quantities and they have not yet been determined with sufficient accuracy to allow use of them in judging the molecular weight. It is felt that the primary amino determinations are likely to be too high and the aminohydroxy determinations may be too low. In particular, it is noted that further manipulation of the hydrolysate (in the isolation of tryptophane, butyl alcohol extraction, etc.) has caused the loss of some non- $\alpha$ -amino compound and the liberation of an

TABLE I  
*Constituents of Gramicidin Hydrolysate*

Constituent	Per cent of gramicidin	N, per cent of total	Equivalents per 11 N atoms	Equivalents per 16 N atoms
$\alpha$ -Amino N.....	10.7	72.6	7.99	10.9
Amino N.....	11.9	80.4	8.84	12.1
Basic groups (titration).....		80.5	8.85	12.1
Acidic " ".....			9.3	12.7
Ammonia.....		1.4	0.16	0.22
Aminohydroxy N.....		4.6	0.51	0.69
<i>l</i> -Tryptophane.....	40.2	37.3	2.05 moles	2.80 moles
<i>d</i> -Leucine (by isolation).....	22.2	16.1	1.8	2.4
Alanine.....	23 -33	25 -35	2.7 -3.9	3.7 -5.3
Hydroxyl group (before hydrolysis).....			0.85	1.1

equivalent amount of ammonia. It appears that the interesting amino component may be a moderately unstable substance.

The composition of gramicidin can be accounted for to a considerable extent. The nitrogen and oxygen content of the formula  $C_{74}H_{106}N_{15}O_{18}$ , for example, is attributable to a polypeptide consisting of three *l*-tryptophane and nine other amino acid groups, of which some are represented by alanine and *d*-leucine and one by an aminohydroxy acid that is not an  $\alpha$ -amino acid. The ratio of the carbon and hydrogen atoms indicates that the constituents of gramicidin, other than tryptophane, are essentially aliphatic in nature.

*Tyrocidine*

Tyrocidine separates from methanol solution as a well defined crystalline hydrochloride. It reacts slowly with nitrous acid, up to 10 per cent of the nitrogen being liberated in 1 to 2 hours. On electrometric titration the major dissociation, equivalent in amount to the chloride content, is found at around pH 8.6. A secondary dissociation begins to appear above pH 10. These results suggest the presence of basic amino groups in tyrocidine. Titration in formaldehyde reveals an acidity equivalent to the chloride content. In alcohol solution an additional weak acidity is titrated which may be due to very weak carboxyl groups, or more probably to phenolic groups, since the acetyl derivative is neutral.

In one tyrocidine hydrolysate about 73.4 per cent of the nitrogen was present as  $\alpha$ -amino acid nitrogen, 13.3 per cent as ammonia, and 5.9 per cent as tryptophane indole nitrogen. Tyrosine and dibasic amino acid (in part aspartic acid) are present as well as tryptophane. Little is known as yet about the other  $\alpha$ -amino acids or the constituents accounting for the remainder of the nitrogen (about 7.4 per cent). In a study with Lipmann and Dubos (6) it has been shown that about 20 per cent of the  $\alpha$ -amino acids is *d*-amino acid. Tyrocidine is not hydrolyzed by trypsin, pepsin, or papain.

The chloride content has been determined carefully with a view to using it in the estimation of a minimum molecular weight. The best analyses (average 2.76 per cent) indicate an equivalent weight of  $1285 \pm 50$ . The empirical formula which is most satisfactory from the standpoint of the present data is  $C_{63}H_{83}N_{13}O_{13} \cdot HCl$  (mol. wt. 1267). There are some indications that the molecule is made up of at least two of these units. As is seen from Table II, the molecular quantities of weak acid and ammonia, both determinable with considerable accuracy, would be integral if the molecular unit had 26 (or a multiple of 26) nitrogen atoms. The principal other possibility is that tyrocidine could be crystallizing with an average of one-fourth its amide content (two  $—CO—NH_2$  groups per molecule of 13 nitrogen atoms) hydrolyzed to carboxyl groups. This appears highly improbable in view of the regularity with which the same titration value is obtained with all samples of tyrocidine, from the first recrystalliza-



tion to the fifth or sixth. If crystallization has the same implications with molecules of this size that it has with smaller ones, then the excellent crystallizability of the hydrochloride also indicates that it is a chemical individual.

When determined directly on a sample of the polypeptide, tryptophane values are higher and ammonia values lower than those found for a 24 hour acid hydrolysate. The former values appear preferable. The other data show that for 26 nitrogen

TABLE II  
*Constituents of Tyrocidine Hydrochloride*

	Constituent	Per cent of tyrocidine hydrochloride	N, per cent of total	Equivalents per 13 N atoms
Intact tyrocidine hydrochloride	Chloride	2.76		1.03
	Weak acidic group			0.49
	Amino N, 3 min.	0.25	1.7	0.2
	" " 30 "	0.95	6.8	0.9
	" " 140 "	1.5	10.3	1.3
	Tryptophane	16.6	15.9	1.03 moles
Acid hydrolysate of tyrocidine	Ammonia, brief hydrolysis		12.0	1.56
	$\alpha$ -Amino acid (corrected)		73.4	9.5
	Ammonia		13.3	1.73
	Basic groups (titration)		88.6	11.5
	Acidic groups (titration)			12.3
	Dicarboxylic acid		16.5	2.1
	Tryptophane	12.2	11.7	0.76 mole

atoms in the hydrolysate there are twenty-three to twenty-four basic groups (3 ammonia, 19  $\alpha$ -amino nitrogen atoms, 1 to 2 others). 2 indole nitrogen atoms are present as tryptophane. Of the dibasic acid fraction approximately one-fourth of the nitrogen reacts as aspartic acid nitrogen, giving 2 moles of carbon dioxide with ninhydrin. The  $\alpha$ -amino nitrogen value presented in Table II for the whole hydrolysate has been corrected for this effect. The quantity of acidic groups recovered appears to be in excess of that to be expected if the hydrolysis were of  $\text{—CO—NH—}$

linkages alone; however, more determinations must be performed on tyrocidine hydrolysates before the facts will be clear.

According to the evidence given above, tyrocidine appears to be a polypeptide of about twenty amino acid residues (including tryptophane, tyrosine, and aspartic acid), combined in such a way that two basic amino groups, three amide groups, and one carboxyl or acidic phenolic group are free.

#### EXPERIMENTAL

*Materials and Methods*—The gramicidin and tyrocidine hydrochloride samples which were used had been recrystallized four or more times.

Where in this paper quantitative results are expressed with limits of variation, the  $\pm$  increment indicates the maximum experimental variation, and not the probable error.

Nitrogen was determined by a micro-Kjeldahl method under conditions which allowed quantitative recovery of tryptophane nitrogen (see (2)). Ammonia was determined by titration after steam distillation in the presence of calcium hydroxide. In some cases vacuum distillation at a lower temperature was used. Amino nitrogen was determined by the nitrous acid method of Van Slyke (13).  $\alpha$ -Amino nitrogen of amino acids (" $\alpha$ -amino" or "carboxyl nitrogen") was calculated from the carbon dioxide produced in the quantitative ninhydrin reaction, carried out at pH 4.7, according to the procedure of Van Slyke and Dillon (9). Titrations of basic groups in 90 per cent acetone (14) and of acidic groups in 90 per cent alcohol (15) were conducted in duplicate, each in comparison with color standards at two intensities of color. In the case of the alcohol titrations, since electrolytes affect the thymolphthalein color, sodium chloride was added to provide an excess of neutral electrolyte in all samples. Chloride was determined by the Volhard titration. Tryptophane was determined by a colorimetric method with *p*-dimethylaminobenzaldehyde (16); visual comparison was made at a series of concentrations in a range in which standard tryptophane solutions gave color densities proportional to concentration.

*Chemical Properties of Gramicidin*—Gramicidin shows no amino groups reactive with nitrous acid or ninhydrin, and no basic or acidic groups titratable in acetone or alcohol solution. Micro-

analysis reveals no sulfur, halogen, methoxyl, O- and N-acetyl, or ash.

On quantitative acetylation with acetic anhydride and pyridine by the method of Stodola (17), the relative weights of gramicidin required to accept 1 equivalent of acetyl were 1200, 1300, 1140, 1240. The average (1220) should possibly be corrected to 1150, inasmuch as the usual acetylation is not quite complete (glucose 98, resorcinol 94 per cent of theoretical). The gramicidin was entirely dissolved and there was no apparent decomposition. It should be pointed out that the result would not be affected by small amounts of moisture, also that even had there been acetolysis of peptide bonds, there might be no effect on the result, since 1 equivalent of acid should be liberated for every acetyl residue bound.

Gramicidin, dissolved in camphor, brought about a lowering of the freezing point, indicating molecular weights of 1540, 1390, and 1385. Molecular weights of known substances obtained similarly were *p*-bromoacetanilide (mol. wt. 214) 212, benzoic acid (mol. wt. 122) 127,  $\beta$ -pentaacetylglucose (mol. wt. 390) 379. Several readings of the freezing point were made with each camphor solution, the mixture being removed from the bath whenever the temperature or rate of heating was being readjusted. One mixture was overheated somewhat and became gradually brownish—the depression of the freezing point in this solution gradually became greater, eventually corresponding to a molecular weight of 1260, a figure not considered significant.

The elementary analysis of gramicidin (2) allows calculation of a number of approximate empirical formulas in the range of molecular weight suggested above; for example,

Found.....	C 62.7, H 7.59, N 14.8
Calculated. $C_{50}H_{72}N_{10}O_9$ (957).....	" 62.75, " 7.58, " 14.64
" $C_{55}H_{79}N_{11}O_{10}$ (1054).....	" 62.65, " 7.55, " 14.62
" $C_{60}H_{86}N_{12}O_{11}$ (1151).....	" 62.6, " 7.53, " 14.60
" $C_{74}H_{108}N_{15}O_{13}$ (1413).....	" 62.9, " 7.49, " 14.87
" $C_{79}H_{112}N_{16}O_{14}$ (1510).....	" 62.85, " 7.48, " 14.84
" $C_{78}H_{112}N_{16}O_{14}$ (1498).....	" 62.55, " 7.54, " 14.96

*Hydrolysate of Gramicidin. Quantitative Study*—All the data reported here were obtained from hydrochloric acid hydrolysates. Loss of tryptophane on acid hydrolysis of proteins is probably

due largely to condensation with aldehydic substances and is accompanied by formation of deeply colored products. Strongly acid hydrolysates become considerably darker in color when exposed to air, particularly at low temperatures at which the solubility of the gases is higher. Accordingly all hydrolyses were carried out under a carbon dioxide atmosphere.

Gramicidin (2.3 gm.) was dissolved in acetic acid (10 cc.) and there was added the maximum amount of 6 N hydrochloric acid (8 cc.) that could be put in without precipitation of gramicidin as a viscous oil. The mixture was boiled under a reflux with a stream of carbon dioxide passing over the solution, and at 30 minute intervals more 6 N hydrochloric acid was added (precipitation being avoided). The acetic acid concentration had been reduced to 8.5 per cent by volume at  $3\frac{1}{2}$  hours and further additions were not made (total volume 118 cc.). Hydrolysis under these conditions is rapid and is completed in about 24 hours, with the production of only slight yellow coloration. Pure tryptophane, similarly treated, is only about 0.7 per cent destroyed, according to the carboxyl nitrogen determinations, and is not appreciably racemized. The hydrolysates were evaporated to dryness *in vacuo* three times under a stream of carbon dioxide, to remove excess hydrochloric acid.

Colorimetric analysis of a newly prepared hydrolysate of gramicidin indicated tryptophane equivalent to 37.3 per cent of the total nitrogen. Analyses of older samples have averaged about 35 per cent tryptophane nitrogen. The higher value is chosen as more nearly correct.

Free ammonia in gramicidin hydrolysates was always so small in amount (0.8 to 1.4 per cent of the total nitrogen) that it is safe to consider it as only a side product. The quantity of amino nitrogen reported in Table I has been corrected for one-fourth the amount of ammonia, since about this proportion of ammonia will react with nitrous acid in the time used for the reaction with amino acids (13). The correction amounts to less than 0.5 per cent of the total amino nitrogen. The number of acidic and basic groups was calculated from the alcohol and acetone titrations and the chloride content (retained hydrochloric acid).

The quantity of ammonia found after periodate acted upon a hydrolysate from which tryptophane had been removed was 0.8

mole, per mole of periodate reduced to iodate. This is evidence that the glycol grouping oxidized is a 1-amino-2-hydroxy grouping. After treatment with periodate the amino nitrogen was decreased while the  $\alpha$ -amino nitrogen remained unaffected. For example, in one hydrolysate freed of tryptophane, which contained, per cc., 0.330 mg. of amino and 0.308 mg. of  $\alpha$ -amino nitrogen, there were found, after reaction with periodate and addition of glucose to destroy the excess reagent, 0.310 mg. of amino and 0.309 mg. of  $\alpha$ -amino nitrogen. The destruction of amino nitrogen was caused by periodate, not by iodate or iodide. The evidence then shows that the 1,2-aminohydroxy compound is not an  $\alpha$ -amino acid.

TABLE III  
*Theoretical Distribution of Nitrogen in Gramicidin*

Total No. of N atoms	Per cent total N		
	Tryptophane	$\alpha$ -Amino	Amino
10	40.0	70.0	80.0
11	36.4	72.7	81.8
12	33.3	75.0	83.3
15	40.0	73.4	80.0
16	37.5	75.0	81.2
17	35.3	76.5	82.4
Found . . . . .	37.3 (35)	72.6	80.4

The analytical value in parentheses was obtained from hydrolysates that had not been freshly prepared.

Alanine was estimated by deaminating the hydrolysate according to the method of Kendall and Friedemann (8), then determining lactic acid according to the modification of their method by Wendel (18).

On the basis of the most probable integral quantities of components of gramicidin deduced from Table I, the theoretical nitrogen distribution can be calculated for various molecular units considered above (Table III).

*Hydrolysate of Gramicidin. Qualitative Study*—Tryptophane was isolated by mercuric sulfate precipitation of 400 cc. of a hydrochloric acid hydrolysate of gramicidin which contained 720 mg. of tryptophane by colorimetric analysis. The mercury precipitate

was washed and decomposed with barium hydroxide and hydrogen sulfide, yielding a solution having 52 per cent of its nitrogen as amino nitrogen, and containing 650 mg. of tryptophane. By extraction with butyl alcohol, 280 mg. of crystalline tryptophane were recovered. After one recrystallization from water 198 mg. were obtained with the properties of pure *l*-tryptophane.

Found. C 64.9, H 5.95, N 13.57;  $[\alpha]_D^{25} = -30^\circ$  (1% in water)  
Theoretical. " 64.7, " 5.93, " 13.72

The rotation given for *l*-tryptophane in the literature varies from  $-28^\circ$  to  $-33^\circ$ ; recent determinations give  $-32.1^\circ$  (19) and  $-31.3^\circ$  (20).

Leucine was isolated as the  $\beta$ -naphthalenesulfonate (7). The dried salt had the expected nitrogen content (theory, 4.13; found, total N 4.17, amino N 4.15,  $\alpha$ -amino N 4.17). On decomposition of this salt (290 mg.) with pyridine and recrystallization of the product from water, there were obtained 56 mg. of crystalline *d*-leucine:

Found. C 55.0, H 9.96, N 10.70;  $[\alpha]_D^{25} = -14^\circ$  (0.6% in 20% HCl)  
Theoretical. " 54.95, " 9.99, " 10.68

The optical rotation of *d*-leucine is, from the literature, about  $-15.5^\circ$  (21, 22) in 20 per cent hydrochloric acid. The yield of free *d*-leucine (50 per cent) was comparable with that reported by Bergmann and Stein for *l*-leucine (59 per cent;  $[\alpha]_D^{24} = +15.3^\circ$  in HCl) recovered from large quantities of *l*-leucine  $\beta$ -naphthalene-sulfonate (7). It is concluded that the leucine present in gramicidin is pure *d*-leucine and that a small part of it has been racemized during hydrolysis.

Formaldehyde was detected in the periodate-treated hydrolysate (best in a tryptophane-free fraction) by preparation of the dimedon derivative (23). The melting point of the derivative obtained was  $188-191^\circ$  (micro hot stage, uncorrected) and that of the pure formaldehyde derivative  $189-191^\circ$ ; the melting point of a mixture of the two was  $189-191^\circ$ .

When treated with periodate followed by *p*-nitrophenylhydrazine, the hydrolysate yielded a hydrazone soluble in aqueous sodium carbonate. Glyoxylic acid *p*-nitrophenylhydrazone (which would have been formed from serine or isoserine) could not be

isolated, but there was obtained a trace of a reddish brown acidic hydrazone which has not been identified as yet.

Fatty acids or alcohols, basic amino acids, proline, or tyrosine was not detected in gramicidin hydrolysates (small quantities of fatty acid had been obtained from the first hydrolysates studied (1) but these were no longer found when the gramicidin was thoroughly freed of waxy impurities). If the terminal amino and carboxyl groups of the peptide chain in gramicidin are combined as acyl and ester derivatives respectively, then the hydrolysate should contain an aliphatic acid and alcohol. Ether extracts showed no appreciable non-volatile substances, and no evidence of volatile ones other than the acetic acid used in hydrolysis. Since in separate determinations acetyl and methoxyl groups were not found, the possibility remains that gramicidin is a cyclic peptide and that this is the reason for its having no free amino or carboxyl groups.

*Chemical Properties of Tyrocidine Hydrochloride*—Gravimetric determination of chloride in tyrocidine hydrochloride gave 2.61, 2.69, 2.78, and 2.76 per cent (the author is indebted to Mr. A. Elek for the gravimetric analyses). Chloride was also determined by Volhard titration after an alcoholic solution of the salt was precipitated by an excess of dilute nitric acid; the content found was 2.79, 2.87, and 2.83 per cent. The average value 2.76 per cent, corresponds to 1 chlorine atom per 1285 of molecular weight (or 2 atoms per 2570).

When electrometrically titrated with the use of a glass electrode, tyrocidine hydrochloride utilized alkali equivalent to its chloride content, maximum buffering occurring around pH 8.6. Precipitation occurs at pH 8 and above. A second hydrogen dissociation began to occur above pH 10. Formol titrations of the hydrochloride indicated equivalent weights of 1195, 1205. In alcohol solution, in which weakly acidic groups are also titrated, the salt utilizes 1 equivalent of alkali per  $855 \pm 10$  of molecular weight (3 per 2560).

The reagents for the nitrous acid determination of amino nitrogen precipitate tyrocidine. However, the results are consistent and fairly reproducible; those presented in Table II are typical. Tyrocidine gives a strong color reaction with ninhydrin but no

carbon dioxide is liberated; therefore the amino group is free but not in the  $\alpha$  position with respect to a free carboxyl group.

Warming tyrocidine in the presence of concentrated hydrochloric acid and *p*-dimethylaminobenzaldehyde allowed a direct colorimetric analysis for tryptophane. Varying quantities of the hydrochloride showed 16.9, 16.6, 16.3 per cent tryptophane.<sup>1</sup> Ammonia was estimated separately after short hydrolysis in alcoholic hydrochloric acid.

Tyrocidine gives a red coloration with diazobenzenesulfonic acid before and after hydrolysis. Tyrosine was identified by the Millon reaction and by isolation; quantitative determination has not yet been attempted.

The analytical data (2) are in agreement with an empirical formula  $C_{63}H_{83}N_{13}O_{13} \cdot HCl$  (mol. wt. 1267) or its multiples.

Found.        C 59.6, H 6.66, N 14.31, Cl 2.76

Calculated. " 59.7, " 6.68, " 14.37, " 2.80

*Hydrolysate of Tyrocidine*—A tyrocidine hydrolysate was prepared in the same way as for gramicidin. However, the carbon dioxide stream was accidentally interrupted during the hydrolysis, and there was a moderate amount of colored matter produced. Consequently the tryptophane values found are probably too low. All constituents were determined as described above.

The dicarboxylic acid fraction, isolated by twice precipitating the barium salts with alcohol, showed 1.9 acidic groups per atom of nitrogen. Ninhydrin liberated 1.27 moles of carbon dioxide per equivalent of nitrogen, suggesting that one-fourth of the dibasic acid was aspartic acid and the remainder was another dicarboxylic- $\alpha$ -amino acid.

*Acetylation of Tyrocidine*—0.55 gm. of tyrocidine hydrochloride was dissolved in 5 cc. of dry pyridine and treated with 1.5 cc. of acetic anhydride. The solution, which rapidly became precipitable by water, was allowed to stand at 25° for 24 hours. The product was precipitated by addition of ether and reprecipitated

<sup>1</sup> The author has learned by personal communication that Christensen, Edwards, and Piersma find a much lower tryptophane content in tyrocidine hydrochloride. Their method of determination differs from ours, but there is no other explanation at present for the apparent discrepancy.



twice from alcohol solution by ether. The acetylated product (0.5 gm.) is more soluble in alcohol and much less soluble in water than the starting material. The analysis suggests that about three acetyl groups have been introduced for every 13 nitrogen atoms:

Found. C 60.9, H 6.7, N 13.4, acetyl 9.9

The specific rotation is  $[\alpha]_D^{26} = -120^\circ$  (0.7 per cent in absolute alcohol).

The acetyl derivative is not acidic when titrated in alcohol and does not give a blue color with ninhydrin.

*Action of Proteolytic Enzymes*—Crude trypsin, pepsin, papain, and papaya latex were tested for their effect upon tyrothricin and upon partially purified tyrocidine and gramicidin. The mixtures were incubated at  $37^\circ$  and tested by titrations in acetone and alcohol. In no instance was liberation of amino or carboxyl groups detected.

The amount of polypeptide in each titration sample was about 50 mg. Trypsin was used at pH 7, 8, and 9, papain at pH 4.4, 5.6, and 6.4, and pepsin at pH 1.9, 3.0, and 3.9. Papain and papaya latex enzyme were activated with cysteine. The polypeptides were either suspended in water or dissolved in alcohol or glycerol and diluted to 5 per cent alcohol or 20 per cent glycerol. Suitable gelatin and casein substrates were hydrolyzed rapidly under the same conditions.

#### SUMMARY

Gramicidin is a polypeptide which contains no free amino or carboxyl groups. The total nitrogen and oxygen content is accounted for by  $\alpha$ -amino acids and a 1,2-aminoalcohol compound which is not an  $\alpha$ -amino acid. Amino acids which have been identified are *l*-tryptophane, *d*-leucine, and alanine. Several alternative empirical formulas for gramicidin can be proposed. The data have been discussed in connection with two satisfactory formulas corresponding to molecular weights of 1054 and 1413.

Tyrocidine hydrochloride is a salt of a polypeptide having free basic amino groups. The most probable molecular unit has two amino groups, three amide groups, and one weakly acidic carboxyl or phenolic group and a molecular weight of 2534. Tryptophane, tyrosine, and dicarboxylic amino acids, in part aspartic acid, have been detected.

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## THE COMPOSITION OF GRAMICIDIN AND TYROCIDINE\*

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This communication and the preceding one by Hotchkiss (1) are accounts of independent investigations on the nature of gramicidin and tyrocidine hydrochloride. These substances have been isolated, according to the procedure of Hotchkiss and Dubos (2), from the alcohol-soluble bactericidal material synthesized by a strain of *Bacillus brevis* discovered by Dubos (3). We are greatly indebted to Dr. Dubos for a culture of this organism.

Gramicidin, thrice crystallized from acetone, yielded 62.5 per cent carbon, 7.5 per cent hydrogen, 14.6 per cent nitrogen (Dumas method). No sulfur or phosphorus could be detected. The homogeneity of the material is indicated by the constancy of bactericidal activity, of solubility in acetone, and of chemical composition. Injected intraperitoneally into mice simultaneously infected intraperitoneally with 10,000 minimum fatal doses of Type I pneumococcus, 0.2  $\gamma$  of the gramicidin protects more than 50 per cent of the animals. 2 mg. per kilo per day intravenously killed two dogs in 2 and 3 days respectively. The substance lost no bactericidal activity during 2 hours of boiling in alcohol, little during 15 hours of standing in 50 per cent alcohol solution *N* in sodium hydroxide. The loss of activity was 90 per cent complete in 2 hours of boiling in 50 per cent alcohol 0.1 *N* in HCl, and complete in 2 hours of boiling in 50 per cent alcohol *N* in HCl or 0.1 *N* in NaOH. Our gramicidin was highly insoluble in water; when al-

\* Because of the necessity of terminating the investigation this work is reported at the present time. The similarity in the courses pursued by this laboratory and by Dr. Hotchkiss was not recognized until recently. Because of their independent and, in the main, mutually confirmatory nature, these papers are presented simultaneously.

cohol solutions were added to water, flocculation was very rapid. The bactericidal activity *in vivo* showed wide variations with the state of dispersion in water.

Tyrocidine hydrochloride has been isolated according to the procedure of Hotchkiss and Dubos (2), with four crystallizations from alcohol containing hydrogen chloride. We have found 59.7 per cent carbon, 6.9 per cent hydrogen, 14.5 per cent nitrogen (Dumas), and 2.52 per cent chlorine (Patterson (4)). Tyrocidine hydrochloride showed a solubility, constant on repeated extraction with ethanol 0.1 N in HCl, of 5 mg. per gm. of solution. About 5  $\gamma$  of our tyrocidine hydrochloride were required to protect 50 per cent of mice infected with 10,000 minimum fatal doses of Type I pneumococcus, under the same conditions as for gramicidin. With the lapse of time between infection and treatment the activity of tyrocidine dropped off more rapidly than that of gramicidin. Thus only one in ten mice infected with 1000 doses of Type I pneumococcus survived when treated 10 minutes later with 50  $\gamma$  of tyrocidine hydrochloride, whereas six out of ten mice survived when treated 10 minutes after infection with 25  $\gamma$  of gramicidin. 10 mg. per kilo per day of tyrocidine hydrochloride in aqueous suspension injected intravenously killed two dogs in 1 and 2 days respectively.

*Components of Gramicidin and Tyrocidine Molecules*—The presence of tryptophane in gramicidin, and of tryptophane and tyrosine in tyrocidine hydrochloride, was reported by Hotchkiss and Dubos (5). Their data suggest that the two substances are polypeptides, containing in the vicinity of ten amino acids per molecule. No groups dissociating or associating hydrogen ions were observed in gramicidin. A group dissociating near pH 8.5 was reported for tyrocidine (2).

In gramicidin we found no evidence of dissociating groups in 60 per cent alcohol when titrated with acid or alkali. The observation that "graminic acid" formed a chloroplatinate insoluble in ethanol led us to observe, by titration, that the polypeptide behaved as a base. Following the report of the basic nature of "graminic acid" and the renaming of the substance "tyrocidine hydrochloride" by Hotchkiss and Dubos (2), we were able to confirm the isolation procedure described by them for tyrocidine hydrochloride. Titration of tyrocidine hydrochloride in aqueous suspension showed a

group dissociating 1 hydrogen ion in the vicinity of pH 9 in a quantity representing approximately 14 equivalents of nitrogen. The presence of an additional group dissociating above pH 11 was suggested.

Only small amounts of nitrogen were released from either polypeptide by the action of nitrous acid for 15 minutes by the Van Slyke technique. The substances were in a flocculated state, so that the reaction may have proceeded slowly; in the time interval employed there was little evidence for the presence in tyrocidine of an  $\alpha$ -amino group equivalent to the alkaline dissociation constant observed by electrometric titration.

#### EXPERIMENTAL

50 mg. of gramicidin in 1.2 ml. of 60 per cent EtOH were titrated with 0.1 N HCl and 0.1 N NaOH in the same solvent by a glass electrode at 25°. 60 per cent alcohol was titrated under identical circumstances. The pH values reached were as follows:

Reagent added, ml.....	0.00	0.05 HCl	0.15 HCl	0.05 NaOH
Gramicidin in 60% EtOH.....	5.86	2.78	2.25	11.3
60% EtOH alone.....	6.81		2.23	11.3

50 mg. of tyrocidine hydrochloride were suspended in 1.5 ml. of water and titrated with 0.1 N NaOH by glass electrode, with 5 minutes of stirring between the alkali addition and pH determination. The  $\alpha$ -curve for this titration is shown in Fig. 1.

6.00 mg. of gramicidin, introduced into the Van Slyke apparatus in glacial acetic acid solution, yielded 0.2 per cent of its nitrogen by the action of nitrous acid for 15 minutes at 25°. Tyrocidine hydrochloride, 6.02 mg., and the crude bactericidal material (Lot 80, no gramicidin or tyrocidine removed), 4.82 mg., yielded, respectively, under the same conditions 1.33 and 1.24 per cent of their nitrogen contents.

#### *Examination of Hydrolysis Products of Gramicidin and Tyrocidine*

Hydrolysis of tyrocidine by boiling 20 per cent aqueous hydrochloric acid proceeded rapidly, the material passing into solution in about 2 hours. Gramicidin required about 18 hours contact

with boiling 20 per cent HCl for complete dissolution. The hydrolyses were continued 18 hours after all material was dissolved, at which time amino nitrogens were maximal. Aqueous sodium hydroxide in sealed alkali-resistant tubes (Corning No. 728) effected complete hydrolysis of tyrocidine at 105° in 24 hours, dissolution requiring an hour. Without constant agitation aqueous alkali attacked gramicidin very slowly. Alcoholic potassium hydroxide hydrolyzed either substance at 105° in 24 hours. Trypto-

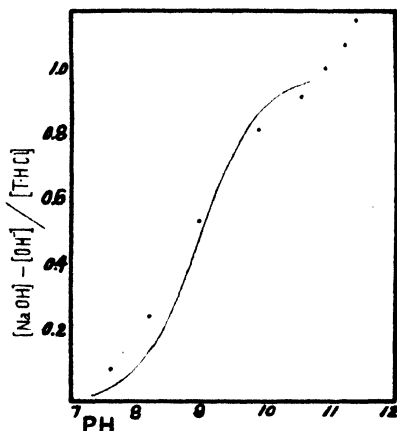


FIG. 1.  $\alpha$ -Curve for the titration of tyrocidine hydrochloride in aqueous dispersion.  $[\text{T} \cdot \text{HCl}]$  is the concentration of tyrocidine hydrochloride in equivalents per kilo of water as indicated by the HCl content. The solid curve is the theoretical form, and the isolated points are the experimental results. Corrections have been made for the effect of sodium ion on the glass electrode.

phane and tyrosine values for tyrocidine were no different whether hydrolysis was performed by alcoholic or aqueous alkali. For all other analyses, and for all isolations, hydrolysis by 20 per cent aqueous HCl was employed.

*Release of Amino Groups by Acid Hydrolysis*—A 42 hour hydrochloric acid hydrolysate of gramicidin, containing 0.194 mg. of N per ml., yielded in 3 minutes with nitrous acid 0.139 mg. of amino N per ml. A 36 hour tyrocidine hydrolysate by hydrochloric acid, containing 0.181 mg. of N per ml., yielded 0.154 mg. of amino N per ml. In the presence of potassium iodide (Kendrick and Hanke (6)) 0.148 mg. of amino N per ml. was found.

*Tryptophane and Tyrosine*—10 mg. portions of gramicidin were hydrolyzed by 6 N alcoholic KOH in sealed tubes at 105°. After acidification by HCl each hydrolysate was made to 25 ml. and 2 ml. aliquots taken for tryptophane analyses by the method of Bates (7) with dimethylaminobenzaldehyde, and by the method of Folin and Marenzi (8).

100 mg. samples of tyrocidine hydrochloride were hydrolyzed similarly, acidified with sulfuric acid, and made to 10 ml. Aliquots were taken for (a) tryptophane and tyrosine determinations according to Folin and Marenzi (8), (b) determination of total phenolic substances according to Folin and Marenzi, (c) trypto-

TABLE I  
*Tyrosine and Tryptophane Analyses*

	Tyrosine, Folin and Marenzi	Tryptophane		Substances reducing phenol reagent, as tyrosine
		Folin and Marenzi	Bates	
	per cent	per cent	per cent	per cent
Tyrocidine HCl, hydrolyzed by alcoholic NaOH.....	13.29	5.00	5.91	18.84
Tyrocidine HCl, hydrolyzed by aqueous NaOH.....			5.94	18.8
Tyrocidine chloroplatinate.....			7.19*	
Gramicidin.....	0.00	35.2	33.6	

\* Expressed as per cent tryptophane in tyrocidine hydrochloride, to permit comparison.

phane determination by the method of Bates. 6 N aqueous sodium hydroxide was also employed for the hydrolysis of tyrocidine.

Tryptophane analyses were also performed on a carefully prepared sample of tyrocidine chloroplatinate. A portion of tyrocidine hydrochloride was isolated with three crystallizations from large volumes without heating to above 35°. At a concentration of 1 per cent in ethanol, the chloroplatinate of this was precipitated. Dried at 100° *in vacuo*, this contained 10.1 per cent nitrogen.

The tryptophane and tyrosine contents of these preparations are shown in Table I.

*Isolation of Tryptophane*—1 gm. of gramicidin was hydrolyzed by 25 cc. of 20 per cent HCl for 36 hours. The solution was freed



of excess HCl *in vacuo*, made to 100 ml., and decolorized by 300 mg. of norit A. The stock solutions so prepared were designated Hydrolysate A. From one-half of this solution tryptophane-mercuric sulfate was precipitated and tryptophane freed of mercury and sulfate (9). The mother liquor from this precipitation was also freed of mercury and sulfate, and concentrated and designated Solution B. The tryptophane solution upon electrometric titration showed pK 2.4 and 9.4. Upon concentration crystals separated,  $[\alpha]_D^{20} = -30^\circ$ , 13.6 per cent N, 6.80 per cent amino N.

*Acetyl Groups*—No steam-volatile acids could be recovered from hydrolysates by aqueous sodium hydroxide of gramicidin and tyrocidine hydrochloride.

*Fatty Acids*—No ether-soluble fatty acids could be recovered from hydrolysates by alcoholic potassium hydroxide of gramicidin or tyrocidine.

*Detection and Isolation of Miscellaneous Amino Acids*—By the method of Kapeller-Adler (10), applied to samples of Hydrolysate A, phenylalanine was shown absent from gramicidin and present to the extent of 22 per cent in tyrocidine hydrochloride. A standard was used containing the same quantity of tyrosine and tryptophane as tyrocidine, and the comparison made at 560 m $\mu$ . Saturation of tyrocidine Hydrolysate A at a concentration of 200 mg. per ml. with gaseous HCl yielded crystals, which upon two crystallizations were found to be pure phenylalanine hydrochloride, 6.93 per cent N.

No hydroxyproline was detected in gramicidin or tyrocidine Hydrolysate A by the method of Waldschmidt-Leitz and Akabori (11). No proline was precipitated by ammonium rhodanilate (12) from either hydrolysate.

By the precipitation of leucine 2-naphthol-7-sulfonate (13) from gramicidin Hydrolysate A, from 12 to 23 per cent of *d*-leucine was indicated. A 12 per cent yield was isolated.  $[\alpha]_D^{20} = +11^\circ$  (0.82 per cent solution in water). From gramicidin Solution B, a 16 per cent yield of leucine was isolated by precipitation of the copper salt and crystallization as free leucine, 10.7 per cent N,  $[\alpha]_D^{20} = -14^\circ$  (2.4 per cent solution in 20 per cent HCl).

The presence of about 14 per cent alanine in tyrocidine and about 30 per cent in gramicidin was suggested by the incompletely specific method of Friedemann and Kendall (14).<sup>6</sup> By isolation of alanine

dioxypyridate (15) from gramicidin and tyrocidine hydrolysates (Hydrolysate A) containing approximately 23 mg. of alanine per ml., the presence of about 22 per cent of alanine in gramicidin and 6.6 per cent in tyrocidine hydrochloride was indicated.

1 ammonia molecule for every 13 to 16 nitrogen atoms was released from gramicidin Hydrolysate A by the action of periodate, according to the procedure of Van Slyke *et al.* (16). The presence of a substance with a hydroxyl and an amino group on adjacent carbons was indicated. A trace (about 0.05 per cent) of pre-formed ammonia was found in this hydrolysate. 1.86 per cent ammonia nitrogen was isolated from tyrocidine hydrochloride hydrolysates by aeration, but no additional ammonia was released by periodate. The hydroxyamino compound was not threonine (Block and Bolling (17)); nor was it hydroxylysine or hydroxyglutamic acid, since it was absent in the phosphotungstic acid precipitate and in the barium salts precipitated by alcohol. Upon fractional crystallization at pH 7, it was concentrated in the more soluble fractions. This makes it improbable that the substance is serine or isoserine.

No glycine could be detected in hydrolysates of gramicidin and of tyrocidine by the method of Patton (18).

Gramicidin and tyrocidine hydrolysates were examined for the presence of dicarboxylic acids according to the procedure of Jones and Moeller (19). Gramicidin yielded about 5 per cent of its nitrogen and tyrocidine about 15 per cent of its nitrogen as insoluble barium salts. The material separated from gramicidin yielded no insoluble copper salt, and formed a hydrochloride only moderately soluble in concentrated hydrochloric acid. Electro-metric titration indicated the presence of a monoaminomonocarboxylic acid. This substance was not one of the recognized components of gramicidin.

The barium salt separated from tyrocidine yielded no hydrochloride insoluble in concentrated hydrochloric acid, although a preliminary titration indicated that it was a monoaminodicarboxylic acid.

Precipitation of phosphotungstates according to Cavett (20) led to the recovery of a monoaminomonocarboxylic acid from gramicidin hydrolysates freed of tryptophane. This appeared to be alanine. From tyrocidine hydrolysates freed of ammonia about 15

per cent of the total nitrogen was precipitated by phosphotungstic acid. This fraction has not been examined.

#### DISCUSSION

The evidence obtained up to this time indicates that gramicidin and tyrocidine are polypeptides, and no hydrolysis products atypical to this class of compounds have been isolated, unless the presence of *d*-leucine is considered so. The tryptophane content of gramicidin suggests that the molecule contains a multiple of 6 nitrogen atoms, of which 2 are due to tryptophane, and that the molecular weight is a multiple of about 575. The quantities of tryptophane, leucine, alanine, and the hydroxyamino compound have the approximate molecular ratios 2:2:(2 or 3):1. That the specific activity of gramicidin does not reside solely in these amino acid residues and their linkages to each other is indicated by the loss of activity upon heating with dilute alkali, under conditions which would not split peptide linkages nor destroy any of the recognized components.

The tyrosine content and the chloride content of our tyrocidine hydrochloride are consistent in suggesting that the molecule has 14 nitrogen atoms or a multiple thereof and a molecular weight of approximately 1350 or a multiple thereof. With several of our early preparations of tyrocidine hydrochloride difficulty was encountered in obtaining uniform tryptophane values. Tyrocidine chloroplatinate, however, showed a constant ratio of tryptophane to tyrosine of 1:2, suggesting a minimum molecular weight of about 2700. The molecular ratios of tyrosine, alanine, phenylalanine, ammonia, and dibasic amino acid approximate 1:1:2:2:2.

The suggestions by Dr. Hotchkiss (personal communication) that the gramicidin molecule contains a multiple of 5 nitrogen atoms and that the tyrocidine molecule contains a multiple of 13 nitrogen atoms are not strictly excluded by our data.

#### SUMMARY

Evidence has been presented that gramicidin is a polypeptide including among its components *l*-tryptophane, alanine, *d*-leucine, and a hydroxyamino compound, and that tyrocidine is a polypeptide containing tryptophane, tyrosine, phenylalanine, alanine, a dicarboxylicamino acid, ammonia, and nitrogenous bases pre-

cipitated by phosphotungstic acid. Evidence has been presented for the absence of numerous substances. Analytical data for the above components are given, and the possible molecular size and composition of the polypeptides have been discussed.

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## SOME PROPERTIES OF GRAMICIDIN

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The isolation of a crude but highly bactericidal substance of a non-protein nature from cultures of an aerobic sporulating bacillus was recently announced by Dubos and Cattaneo (1). From this substance, later referred to as tyrothricin, two crystalline compounds, *gramicidin* and *tyrocidine hydrochloride* (2, 3), were obtained. Both compounds were found to be highly bactericidal, *in vitro*, for Gram-positive microorganisms. With a special technique, gramicidin in very small doses protected mice infected intraperitoneally with pneumococci (3), whereas tyrocidine hydrochloride exerted a similar protection at a much higher dose (2, 4). Shortly after the announcement of the isolation of gramicidin (3), Hoogerheide (5) reported the isolation of a crystalline, bactericidal compound termed Fraction II, from cultures of a sporulating bacillus obtained from soil. Although many of the chemical and biological properties of Fraction II are similar to those of gramicidin, Hoogerheide did not regard the two as identical, because of differences in behavior towards certain reagents and because of disagreement of nitrogen analyses.

In our investigations, the isolation of gramicidin from tyrothricin was accomplished by the method originally employed by Hotchkiss and Dubos (3) and also by the method described by Hoogerheide (5).

Gramicidin was also isolated from tyrothricin by a prolonged continuous extraction with absolute ether. This observation is somewhat unexpected, inasmuch as it has been demonstrated that gramicidin is a polypeptide of high molecular weight (3). Owing to the small but definite solubility of gramicidin in ether, the pure

product crystallizes in the ether reservoir during the extraction period.

Gramicidin prepared by the above methods was subjected to repeated recrystallizations with different solvents as a test for its homogeneity. The steps were followed by analyses, by determinations of optical rotation and of bactericidal activity, and also by studies of ultraviolet absorption spectra. The results indicate

TABLE I  
*Physical and Analytical Data of Gramicidin*

Source	M.p.	Analyses*			[ $\alpha$ ] <sub>D</sub>	$E_{1\%}^{1\text{cm.}}$ at 2815 Å.
		Carbon	Hydrogen	Nitrogen		
	°C.				degrees	
Prepared by method of Hotchkiss and Dubos	228-230	62.71	7.36	14.84	+5.5	124
Prepared by Hoogerheide's method	228-230	62.67	7.41	14.88	+5.2	122
Prepared by ether extraction	228-230	62.75	7.38	14.79	+5.5	128
Reported by Hotchkiss and Dubos (3)	228-230	62.7	7.5	13.9	+5	
Reported by Hoogerheide (5) for Fraction II	228-232	62.7	7.52	14.9	Slightly positive	126

The authors wish to acknowledge with gratitude the microanalytical determinations carried out by Mr. D. F. Hayman, Mr. W. R. Reiss, Mr. R. N. Boos, and Mr. H. S. Clark of Merck and Company, Inc.

\* The analyses recorded for our preparations are averages of many determinations on several samples of the same degree of purity. The analytical results reported by Hoogerheide are also average values.

that gramicidin is a single substance, and that its analytical, physical, and biological properties are identical with those of Fraction II.

As indicated in Table I, the reported difference in nitrogen content between gramicidin and Fraction II is non-existent. In agreement with Hoogerheide, we observed that gramicidin takes up about 2 per cent moisture which it retains tenaciously. This factor may account for the lower nitrogen value previously reported for gramicidin.

The ultraviolet absorption spectra of our samples resemble closely those given by Fraction II. As indicated in Fig. 1 the

two compounds absorb in the same regions, showing a principal maximum at 2815 Å. and a second maximum at 2910 Å. Careful examination also reveals some fine structure at 2690 Å. which was not observed by Hoogerheide.

In order to determine quantitatively the activity of samples of gramicidin obtained by fractional crystallization a method was developed which made use of a sensitive strain of micrococcus (MY strain). This organism is Gram-positive and forms a yellow

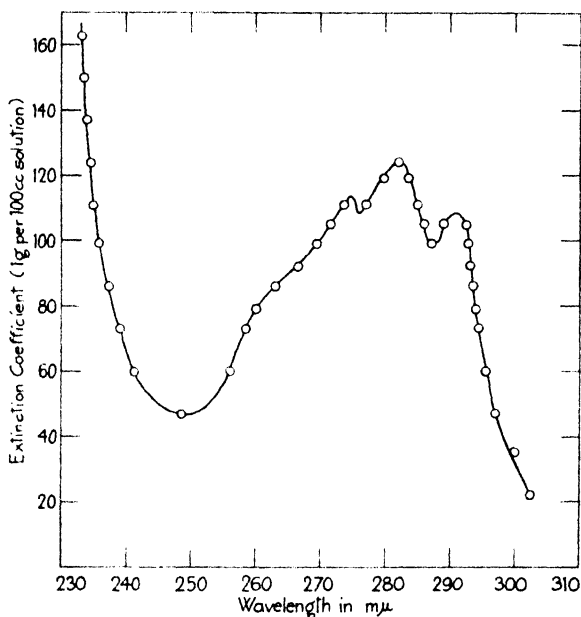


FIG. 1. Absorption spectra of gramicidin

pigment. It produces an acid reaction in dextrose, maltose, and lactose broths, but not in sucrose broth, and also liquefies gelatin and reduces nitrates to nitrites. This particular strain of micrococcus was used because it is sensitive to the action of gramicidin<sup>1</sup> and gives readily reproducible counts in plating experiments.

5 hour cultures of the micrococcus grown under carefully stand-

<sup>1</sup> Many strains of *Staphylococcus aureus* have been found by us and by Dubos (personal communication) to be very resistant to the action of gramicidin.



ardized conditions in order to obtain populations of constant size were used. As a check, direct microscopic counts were made on each culture used in the test. Cultures that showed considerable variation from the normal were discarded. The bactericidal activity of a particular sample of gramicidin was evaluated by determining the amount necessary to destroy 50 per cent of the micrococcus cells present in 1.0 cc. of a 5 hour culture, within 2 hours at 37°. The number of surviving cells was determined by the plating method. A sample of partially purified tyrothricin

TABLE II  
*Quantitative Assay Results*

Substance	Source	Activity*
1. Gramicidin	Ether extraction of tyrothricin	4
2. "	(1) recrystallized six times from acetone	4
3. "	Prepared by method of Hotchkiss and Dubos	5
4. "	(3) recrystallized from butyl alcohol	4
5. "	Prepared by Hoogerheide's method	4
6. "      flavianate	From gramicidin and flavianic acid	6
7. "      "	(6) recrystallized three times from methanol	5
8. "	Solid heated at 111° for 3 hrs.	4
9. "	Alcoholic solution boiled 36 hrs.	4

\* The values given represent the weight, in micrograms of any fraction, necessary to destroy 50 per cent of the bacterial cells within 2 hours at 37°. The results are accurate to  $\pm 1 \gamma$ .

which was known to cause lysis of the cells in the test culture, in amounts of 5  $\gamma$  but not 4  $\gamma$ , was used as a control on the sensitivity of the micrococcus cells and of the test conditions. The values obtained for gramicidin were accepted only when the expected values for the tyrothricin were found.

Some of the results of this quantitative study are given in Table II.

A sample of tyrocidine hydrochloride when tested for activity by the same method as was used for gramicidin gave 50 per cent destruction with 7  $\gamma$ . Gramicidin, therefore, appears to be about twice as active as tyrocidine hydrochloride under our conditions

of testing for activity. The differences in chemical and biological properties of gramicidin and tyrocidine have recently been discussed by Dubos and Hotchkiss (6).

Pure gramicidin responds to the common protein tests in the same way as does Fraction II. Thus it gives the biuret, xanthoproteic, and the Hopkins-Cole tests. The latter reaction takes place slowly. In spite of the fact that gramicidin does not contain free amino or carboxyl groups, it combines with some of the usual protein precipitants. Crystalline products were obtained when gramicidin was brought into contact with flavianic acid or rufanic acid in methyl alcohol. Picric acid, however, produced a gel.

TABLE III  
*Analyses of Gramicidin Flavianate*

The gramicidin flavianate was prepared by mixing a solution of 0.5 gm. of gramicidin in 2.5 cc. of methanol and 0.5 gm. of flavianic acid in 2.5 cc. of methanol. Precipitation occurs after the mixture has stood for a few minutes. The product was always recrystallized from boiling methanol.

No. of recrystallizations	Carbon	Hydrogen	Sulfur
1	60.68	7.00	
2	60.67	7.32	
2	60.78	7.10	
3	60.50	7.00	0.97, 0.97, 0.96

No precipitate was formed when gramicidin was treated with chloroplatinic acid, gold chloride, or phosphotungstic acid.

The flavianate of gramicidin (decomposition at 215–218°), prepared by mixing methyl alcohol solutions of flavianic acid and gramicidin, appears to be a complex which dissociates to an appreciable extent when dissolved in methanol. On recrystallization of this substance from hot methanol about 40 per cent recovery is obtained. When additional flavianic acid solution is added to the mother liquor, flavianate precipitates. That the flavianic acid in the flavianate is part of a true complex and not held by gramicidin by some adsorption phenomena was indicated by the constancy of the analyses of repeatedly recrystallized samples (Table III). In addition, it was found that a sample of the flavianate in absolute methanol (29 mg. per 10 cc.) has an optical density of 0.50

at 4600 Å. This value is higher than the density of the same weight concentration of pure flavianic acid.

The molecular weight of gramicidin, determined by the melting point depression of camphor, has been reported to lie between 1200 and 1600 (2, 5). Our results with this method are in agreement with these values. Inasmuch as the cryoscopic method with camphor as the solvent sometimes leads to erroneous results, a more intensive study of this problem was undertaken.

A determination of the molecular weight was made by a modification of the isothermal distillation method of Barger, Niederl, and collaborators (7). In the determination under consideration, 82.77 mg. of a carefully prepared, rigorously dried sample of gramicidin were weighed into the right-hand limb of a V-tube, and 4.119 mg. of a sample of pure 2,3-dimethyl-1,4-naphthoquinone into the left-hand limb. The gas had been carefully discharged from the entire vessel before the introduction of these materials, and methyl alcohol (over a drying agent) was vacuum-distilled into the two limbs of the vessel, thus forming the solutions. These were frozen at dry ice temperature, and the vessel sealed under a vacuum.

In consideration of the serious errors which would accrue as a result of any appreciable quantity of low molecular weight impurities, a rigorous technique, designed to avoid the presence of water or of atmospheric gases, was used at every stage of the filling operation. The quantities of methanol introduced into the right- and left-hand limbs were respectively 6.78 and 4.51 cc. The vapor pressure of the solvent from the two solutions thus formed is the same, in case the molecular weight of the unknown is 2500. (A preliminary diffusion experiment in which *n*-butanol was used as the solvent, had indicated a value of the molecular weight in the range of 2500 to 4500.) The closed system was kept at a constant temperature (24°), and the transfer of solvent measured from time to time by observing the volume of the solution in each of the two limbs. The results are shown in Table IV.

A graph of the results shown in Table IV indicates that the distillation proceeds uniformly in time until the 70th day. Thereafter there is no distillation, indicating that the two resultant solutions are in equilibrium with respect to the vapor pressure of the solvent. Accordingly, from the data above, this condition of

equilibrium indicates for the molecular weight of gramicidin a value of 3100.

It is interesting to note that the analytical results of gramicidin flavianate are in agreement with this value. On the assumption that 1 molecule of gramicidin combines with 1 molecule of flavianic acid, the sulfur content (0.97 per cent) indicates a molecular weight of  $2986 \pm 300$ ; the carbon ratio between gramicidin flavianate and its individual components places the value at  $3036 \pm 300$ .

An attempt was next made to determine the molecular weight of gramicidin dissolved in phenol by measurement of the freezing

TABLE IV  
*Molecular Weight of Gramicidin As Determined by Isothermal Distillation*

Days	Volume		$\Delta V$ (left limb)*	Apparent mol. wt.
	Left limb	Right limb		
	cc.	cc.	cc.	
0	4.51	6.78	0.0	(2500)
13	4.58	6.70	+0.07	
44	4.81	6.49	+0.30	(2800)
62	5.03	6.25	+0.52	
73	5.10	6.20	+0.59	3100
91	5.10	6.18	+0.59	3100
105	5.12	6.18	+0.61	3100

The parentheses indicate non-equilibrium values.

\* $\Delta V$  is the volume change in the left limb due to distillation of solvent from the right limb.

point depression. The phenol used as solvent, as well as the technique of the measurements, was first checked by using diphenylamine (mol. wt. 169) as a standard. Values of 165 and 166 were obtained under conditions entirely comparable with those used with gramicidin. In the first experiment (1.067 gm. of gramicidin, 12.26 gm. of phenol, observed depression  $0.746^\circ$ , cryoscopic constant of phenol  $7.4^\circ$  per mole per 1000 gm.) a value of 864 was found for the molecular weight. At this point, the drying of the gramicidin was suspected, and, accordingly, a still more rigorous drying procedure was used. A further determination at approximately the same concentration of gramicidin in phenol gave a result of 908.

As a further check on the cryoscopic method, it was decided to determine the molecular weight of gramicidin dissolved in cyclo-

TABLE V  
*Molecular Weight of Gramicidin As Determined by Freezing Point Depression*

Concentration, gramicidin per gm. cyclohexanol	Temperature	Apparent mol. wt.
gm.	°C.	
0.0409	1.44	1220
0.0247	1.035	1026
0.0204	0.945	926
0.0109	0.635	737
0.00961	0.58	712
0.00713	0.47	652
0.00439	0.32	589

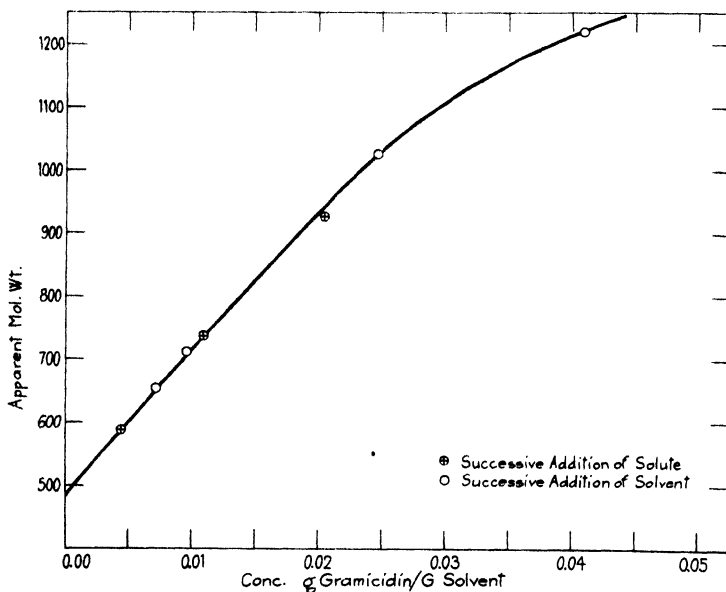


FIG. 2. Molecular weight of gramicidin dissolved in cyclohexanol

hexanol by measurement of the freezing point depression. Eastman, best grade, cyclohexanol was redistilled, the fraction boiling at 161° being retained for use. It froze sharply at 25.15° with only

a  $0.3^\circ$  drift on complete solidification. In view of the scanty and uncertain data in the literature for the value of the cryoscopic constant of this solvent, two determinations of this constant were made. Diphenylamine and benzophenone were used as solutes for this purpose. The series of measurements with diphenylamine indicated that the value of the constant is  $46.4^\circ \pm 1.1^\circ$  (degrees per mole per 1000 gm.), that with benzophenone  $42.7^\circ \pm 0.2^\circ$ . The value  $43.0^\circ$  was adopted for the further determinations. The results obtained are recorded in Table V.

The uniformity of the experimental results is indicated by the graph in Fig. 2. The first series of measurements was made by successively diluting more concentrated solutions, and it was at first thought that the greater specific depressions (and hence the smaller apparent molecular weight) obtained at the greater dilutions was to be attributed to a gradual uptake of moisture in spite of the precautions taken. This explanation had to be abandoned when it was found that the curve in Fig. 2 could be reproduced by successively adding solute to less concentrated solutions.

The anomaly presented by these results remains unexplained. It should be added, however, that the solute recovered from the cyclohexanol solutions was indistinguishable from the starting material.

#### SUMMARY

1. Further characterization of gramicidin, a bactericidal substance, isolated by Hotchkiss and Dubos from cultures of an aerobic sporulating bacillus, are reported.

2. Gramicidin appears to be identical with a crystalline bactericidal fraction isolated by Hoogerheide.

3. Gramicidin forms a crystalline flavianate and rufianate.

4. The molecular weight of gramicidin presents an anomaly in that it appears to depend on the nature of the solvent and the concentration of solute. Cryoscopic determinations in cyclohexanone give values from 600 to 1200 (concentration range 0.4 to 4 gm. per 100 gm. of solvent), whereas isothermal distillation in methanol indicates a molecular weight of 3100. Sulfur and carbon analyses of gramicidin flavianate place the molecular weight of gramicidin as 3000, assuming 1 molecule of gramicidin and 1 molecule of flavianic acid in this complex.

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# CHARACTERIZATION OF THE FUNCTIONAL GROUPS OF BIOTIN\*

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In a previous publication (1) we have described a procedure for the isolation from liver of biotin as the pure crystalline methyl ester. Some of the properties of the pure substance were described, and in a later paper (2) we have given the preparation and properties of free biotin itself. We have also recently reported the results of a series of inactivation experiments on pure biotin which gave some indication of the possible presence or absence of certain types of groups (3). In this present paper we give the results of experiments which by direct chemical attack have led to the recognition of the functional groups present in the biotin molecule. We have obtained evidence that biotin is an N, N'-disubstituted cyclic urea derivative.

As previously reported (1), biotin methyl ester possesses a melting point of 166–167° and is optically active ( $[\alpha]_D^{22} = +57^\circ$  in chloroform). The analytical data are in best agreement with the empirical formula of  $C_{11}H_{18}O_3N_2S$  and show one methoxyl group to be present. The free biotin (2) melts at 230–232°, possesses a rotation of  $[\alpha]_D^{22} = +92^\circ$  in 0.1 N NaOH, and is predominantly acidic in character. Analyses of the compound led to the formula  $C_{10}H_{16}O_3N_2S$  which is in agreement with the results of the analyses

\* The authors wish to express their appreciation to Mr. W. O. Frohring and the Research Staff of the S. M. A. Corporation for the supply of biotin concentrates which made this investigation possible.

† Dr. Hofmann's participation in this work has been made possible by a fellowship granted to him by the Society for Chemical Industry, Basel, Switzerland.

‡ S.M.A. Corporation Fellow.



of the ester. Evidence for the presence of a carboxyl group in biotin has already been presented. The absence of specific absorption bands for biotin in the ultraviolet range suggests the absence of an aromatic ring or of similar structures (2).

Because biotin is inactivated by nitrous acid, it has been assumed that the compound could be an amino acid (4). We have found, however, that no nitrogen is produced when biotin is treated with nitrous acid by the Van Slyke procedure.<sup>1</sup> Furthermore, no color is formed after treatment of biotin with ninhydrin at pH 6.5. The possibility of biotin being an  $\alpha$ -amino acid is consequently eliminated. Inactivation experiments likewise led to this conclusion (3).

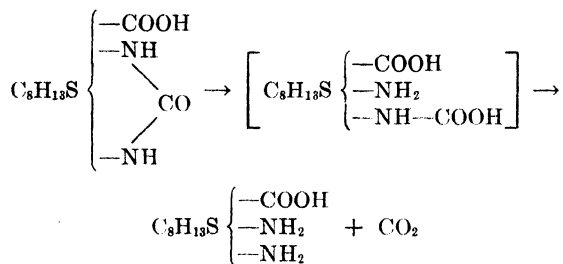
Many other attempts were made to characterize the nitrogen in the biotin molecule. It would be unfruitful, however, to give the many negative experiments in this direction, since the following observation supplied us with the information we desired as to the nature of both of the nitrogen atoms. This same finding also characterized the remaining oxygen atom to be accounted for.

Treatment of biotin or its methyl ester with strong barium hydroxide solution for 20 hours at 140° brings about the formation of a new, optically active compound which can be isolated in 85 per cent yield as the sulfate. The analyses of this compound agree best with the formula  $C_9H_{20}O_6N_2S_2$ . Since one-half of the sulfur in the molecule is present as ionizable sulfate, it appears that the compound is the salt of a substance possessing basic properties. The free compound,  $C_9H_{18}O_2N_2S$ , obtained from the sulfate by treatment with the calculated amount of barium hydroxide, melts with decomposition at 185–190°, and can be sublimed *in vacuo*.

By a micro-Van Slyke procedure an average amino N content of 8.7 per cent was observed for the sulfate of this compound. This indicates the presence of two primary amino groups in the molecule. On benzoylation by the Schotten-Baumann method an alkali-soluble dibenzoyl derivative (m.p. 182–183°) is formed which with diazomethane forms a dibenzoyl methyl ester (m.p. 128–130°). The new compound will be referred to hereafter as the diaminocarboxylic acid.

<sup>1</sup> We are indebted to Dr. Fritz Lipmann for carrying out the micro-Van Slyke analyses by the method of Warburg (5).

The most logical interpretation we can place on the formation of a diaminocarboxylic acid with the loss of 1 carbon atom and 1 oxygen atom from biotin is the cleavage of a cyclic urea derivative. It will be recalled that biotin is predominantly acidic, so much so that biotin crystallizes as the free compound from fairly strong acid solutions. The nitrogen atoms must therefore be extremely weakly basic. However, some basicity is indicated by the fact that the ester can be extracted from chloroform by fairly strong HCl. The conversion of the biotin possessing such weakly basic nitrogen to the much more basic diaminocarboxylic acid fits in with the interpretation offered. The inactivation of biotin by nitrous acid in spite of no liberation of nitrogen gas may possibly be due to the formation of a nitroso derivative, a property of urea derivatives. During the drastic barium hydroxide treatment the urea structure would probably be transformed by the addition of water into the corresponding carbamic acid, which then loses  $\text{CO}_2$  to yield the diaminocarboxylic acid. Therefore, the hydrolytic cleavage of biotin may be written in the following manner.



The urea grouping must be part of a ring system, since no nitrogen and only 1 carbon atom is lost from the biotin by this procedure. The formation of two *primary* amino groups during the hydrolysis indicates further that each of the 2 nitrogen atoms of biotin carries 1 hydrogen atom.

With the characterization of the 2 nitrogen atoms and the 3 oxygen atoms, attention was focused on the nature of the sulfur. It was found that biotin does not contain alkali-labile sulfur and does not liberate  $\text{H}_2\text{S}$  when treated with zinc dust and HCl. After treatment with bromine water no inorganic sulfate could be detected. No positive nitroprusside test was obtained either in

the presence or the absence of sodium cyanide. The stability of the sulfur pointed to a thio ether structure and the experiments which will be described offer support for this assumption.

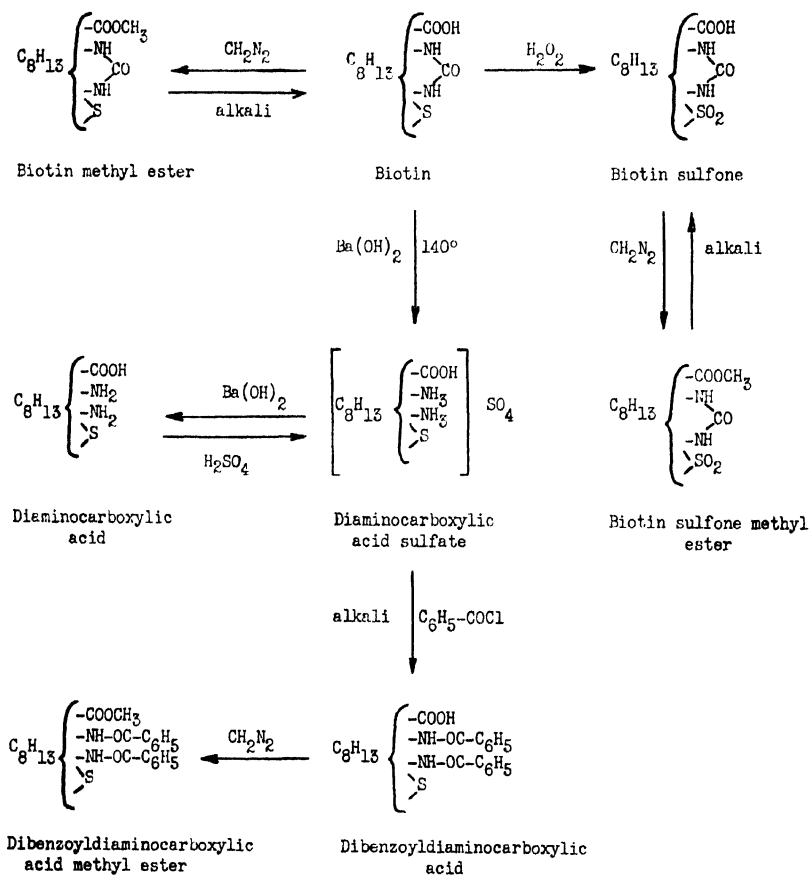


FIG. 1

It had been observed by earlier investigators, as well as by us (3), that biotin is extremely sensitive to peroxides. Accordingly, pure biotin was treated with excess hydrogen peroxide in glacial acetic acid solution at room temperature for 16 to 18 hours. From the reaction mixture it was possible to isolate a crystalline

oxidation product in 90 per cent yield. The analyses of the pure compound, which melts at 274–275° with decomposition, point to the composition  $C_{10}H_{16}O_6N_2S$ , in which 2 atoms of oxygen have been added to the biotin molecule without loss of carbon or hydrogen. The presence of a carboxyl group in the compound is shown by the formation of a methyl ester, m.p. 239–241°, on treatment with diazomethane. The methyl ester is saponified by cold dilute alkali with the formation of the original oxidation product, m.p. 274–275°.

Biotin and the diaminocarboxylic acid when treated with tetranitromethane produce a strong yellow color, in contrast to the new oxidation product which does not produce any color with that reagent. This behavior could parallel the formation of a sulfone from a sulfide, the latter giving strong color, the former remaining colorless when treated with tetranitromethane. Since biotin is not hydrogenated when shaken in the presence of platinum with hydrogen, the molecule apparently does not contain an ethylenic linkage, assuming no poisoning of the catalyst. The sulfur in the thio ether form would therefore seem to be responsible for the color reaction with tetranitromethane. These facts along with the addition of 2 oxygen atoms to the molecule without loss of carbon or hydrogen point to an oxidation by the peroxide treatment of a thio ether to the corresponding sulfone.

The relationships of the diaminocarboxylic acid and of the sulfone to biotin and to the derivatives of all three compounds are shown in Fig. 1.

#### EXPERIMENTAL

*Diaminocarboxylic Acid Sulfate*—10 mg. of biotin or biotin ester were heated in a sealed tube with 1 cc. of water and 200 mg. of barium hydroxide for 20 hours at 140°. The excess baryta was removed with carbon dioxide and the filtrate from the barium carbonate was acidified with 1 N  $H_2SO_4$  until it was faintly acid to Congo red. The precipitated barium sulfate was removed by filtration and the clear filtrate was concentrated *in vacuo* to a small volume. On addition of a few drops of methanol to the solution plate-like crystals appeared. The crystals were collected by filtration and were washed with methanol. The resulting 10 mg. of crystals were further purified by crystallization from a

mixture of water and methanol. The pure material melted at 245–255°,<sup>2</sup> depending upon the rate of heating. It possessed a rotation of  $[\alpha]_D^{22} = -15^\circ$  for a 1 per cent solution in water.

$C_8H_{16}O_6N_2S_2$  (316.4)

Calculated.	C 34.16,	H 6.37,	N 8.85,	S 20.27,	NH <sub>2</sub> -N 8.85,	SO <sub>4</sub> -S 10.13
Found.	" 34.43,	" 6.31,	" 8.54,	" 20.22,	" 8.69,	" 10.07

*Diaminocarboxylic Acid*—10 mg. of the diaminocarboxylic acid sulfate were dissolved in 0.5 cc. of water and 0.64 cc. of 0.1 N barium hydroxide solution was added. The barium sulfate was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The crystalline residue, 5.8 mg. of needles melting at 180–185°, was purified by sublimation *in vacuo* (10<sup>-6</sup> mm.) at 160°. The purified material melted at 186–190° with decomposition.

$C_8H_{16}O_6N_2S$	Calculated.	C 49.52,	H 8.31,	NH <sub>2</sub> -N 12.83
(218.3)	Found.	" 49.33,	" 8.30,	" 14.30

2 mg. of the sublimed material were dissolved in 0.5 cc. of water and the solution was acidified with 1 N H<sub>2</sub>SO<sub>4</sub>. On evaporation *in vacuo* and crystallization of the residue by the addition of methanol, the sulfate was obtained, melting at 245–250°.

*Dibenzoyldiaminocarboxylic Acid*—15 mg. of the diaminocarboxylic acid sulfate were dissolved in 1 cc. of water, and 1 N NaOH was added until the solution was alkaline to phenolphthalein. The solution was cooled with ice. 17 mg. of benzoyl chloride were added, and the solution was shaken for 15 minutes, and kept alkaline by the addition of 1 N NaOH. At the end of this time the alkaline solution was extracted with ether and the ether extracts were discarded. The aqueous layer was acidified to Congo red with 3 N HCl, whereupon the dibenzoyl derivative separated as an oil. The oil was extracted with chloroform. The chloroform solution was washed with water, dried over sodium sulfate, and evaporated to dryness. The crystalline residue was purified by crystallization from a mixture of methanol

<sup>2</sup> The melting points reported herein were determined by the use of the Kofler micro melting point apparatus and are uncorrected.

and ether. The yield of pure compound was 15 mg. of needles melting at 182–183°.

$C_{23}H_{26}O_4N_2S$  (426.5). Calculated, N 6.57; found, N 6.44

*Dibenzoyldiaminocarboxylic Acid Methyl Ester*—10 mg. of the dibenzoyldiaminocarboxylic acid were dissolved in 0.5 cc. of methanol and to this solution was added a freshly distilled solution of diazomethane in ether until the yellow color remained. The solution was kept in the refrigerator for 30 minutes, and was then evaporated to dryness *in vacuo*. The crystalline residue was purified by crystallization from a mixture of methanol and ether. 6 mg. of needles melting at 128–130° were obtained.

$C_{24}H_{28}O_4N_2S$ .	Calculated.	C 65.42, H 6.40
(440.5)	Found.	" 65.46, " 6.36

*Biotin Sulfone*—11.9 mg. of biotin were dissolved in 5.4 cc. of glacial acetic acid and 0.6 cc. of 30 per cent  $H_2O_2$  were added to the solution. The clear solution was kept at room temperature for 18 hours and then evaporated to dryness *in vacuo*. The crystalline residue was dissolved in a few drops of boiling water and the solution was allowed to cool. The biotin sulfone crystallized from the cold solution in long needles. The crystals were removed by filtration and were washed with cold water. The yield of crystals was 7.0 mg., m.p. 274–275° with decomposition. By concentration of the mother liquors an additional 5.0 mg. of the sulfone, m.p. 274–275°, were obtained.

$C_{10}H_{16}O_6N_2S$ .	Calculated.	C 43.47, H 5.84, N 10.13, S 11.61
(276.3)	Found.	" 43.36, " 5.76, " 10.17, " 11.32

The biotin sulfone was esterified in the following manner. 5.0 mg. were suspended in 2 cc. of methanol and the suspension was cooled in an ice bath and treated with an excess of diazomethane for  $\frac{1}{2}$  hour with frequent shaking. At the end of this time the sulfone had dissolved and the yellow color due to the diazomethane remained. The solution was concentrated to dryness *in vacuo* and the crystalline residue, m.p. 238–240°, was sublimed at 220° and  $10^{-5}$  mm. pressure. The sublimate was dissolved in 1 cc. of hot methanol and upon the addition of ether the substance crystallized from the solution. The crystals were

washed with ether and dried. The yield of biotin sulfone methyl ester, m.p. 239–241°, was 4.5 mg. The methoxyl determination indicated the presence of one methoxyl grouping.

Approximately 1 mg. of the biotin sulfone methyl ester was dissolved in a few drops of 2 N NaOH and the solution was acidified with dilute HCl. The crystalline material which separated was washed with water. This material melted with decomposition at 274–275°, the melting point of biotin sulfone.

The authors wish to express their appreciation to Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

#### SUMMARY

Evidence accounting for the functional groups of biotin has been presented. It has been concluded that biotin is a carboxylic acid containing an N,N'-substituted cyclic urea grouping and possessing sulfur in a thio ether linkage.

By alkaline treatment of biotin a sulfur-containing diamino-carboxylic acid containing 1 less carbon atom and 1 less oxygen atom is formed. The sulfone of biotin has been prepared by the oxidation of biotin with H<sub>2</sub>O<sub>2</sub>. The preparations of various derivatives of these compounds have likewise been presented.

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## A RAPID EXTRACTOR FOR URINARY STEROIDS

### II. MODIFICATIONS FOR THE SIMULTANEOUS HYDROLYSIS AND EXTRACTION OF URINE WITH ANY SOLVENT HEAVIER THAN WATER

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Since our publication last year (1), the extractor with a sintered glass disk dispersing carbon tetrachloride has been employed widely in both assay and research laboratories. In addition, the principle of simultaneous hydrolysis and extraction of urine which was suggested has been shown by Talbot, Butler, MacLachlan, and Jones (2) to result in a greatly improved androgen recovery. This, and other work, indicates that considerable time can be saved and a maximum yield of hormone obtained by warming the urine in the extractor with an electrical heater until the equilibrium temperature is reached. With carbon tetrachloride as the extracting solvent this is approximately 60°, and once this temperature is reached the current may be shut off.

The operating temperature can be controlled either above or below 60° by the use of some solvent other than carbon tetrachloride as the extracting medium. A new level is then required in the extractor overflow arm to compensate for the change in specific gravity of the solvent. We have done this by using the sliding cylinder and barrel assembly *B*, shown in Fig. 1. The position of the overflow hole *C* may be adjusted while the extractor is in operation by sliding the plunger *A* up or down through the cork *D* until the porous plate just dips into the urine.

\* Research Fellow on grants from the National Cancer Institute and Eli Lilly and Company to Professor L. F. Fieser.

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With a given dispersion disk the droplet size is dependent on the acidity of the solution and the surface tension of the extracting solvent. Methylene chloride, chloroform, and tetrachloroethylene are all comparable with carbon tetrachloride, while ethylene chloride gives a coarse dispersion.

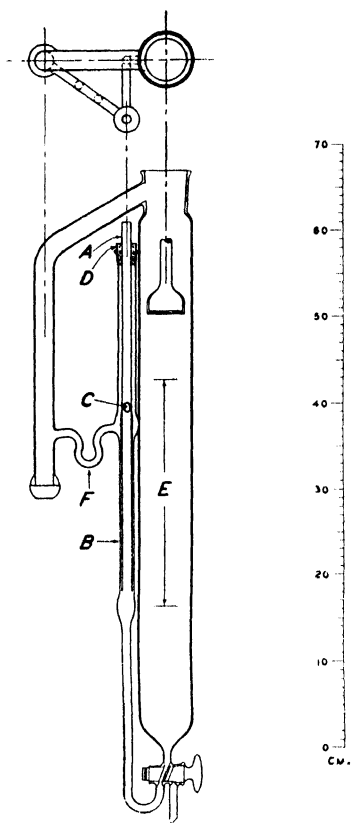


FIG. 1. Extraction apparatus

*Apparatus (Fig. 1)*

*Variable Overflow Device*—The plunger is ground to  $3/8$  inch in diameter and the barrel is made from precision inside ground and polished tubing (Ace Glass, Inc., Vineland, New Jersey), the fit being to a tolerance of from 0.001 to 0.003 inch. The upper sec-

tion of the barrel *B* is 15 mm. outer diameter and the upper section of the plunger *A*, above the overflow hole *C*, is 7.5 mm. outside diameter.

A loosely fitting cork *D* offers sufficient resistance to prevent the plunger from slipping, once a setting has been made.

*Heater*—This occupies the section *E*, which is from 7 to 8 inches long, beginning at a point about 2 inches below the porous disk. To construct the heater two  $\frac{1}{2}$  inch wide stainless steel bands are fastened to each end over strips of asbestos paper 1/16 inch thick and clamped tightly by means of screws and nuts. Then three vertical strips of asbestos paper 1/64 inch thick and 1 inch wide are placed symmetrically around the body of the extractor and wet with water to cause them to stick. A 20 foot length of No. 26 gage resistance wire (4.1 ohms per foot) is then wound on with about  $\frac{1}{4}$  inch spacing between turns and each of the ends is fastened to one of the screws holding the steel bands. The entire winding is covered with a wet piece of 1/16 inch thick asbestos paper and allowed to dry before being used. With the nominal 115 volt current this provides 165 to 175 watts and will warm the contents of the extractor from room temperature to 60° in from 10 to 15 minutes while the extractor is in operation. If the additional heat is not supplied electrically and the heat interchange between the carbon tetrachloride droplets and the urine is relied on to raise the contents of the extractor to the equilibrium temperature, about 1 hour is required to reach the same point. When higher boiling solvents are used, it is sometimes desirable to supply a continuous but small fraction of the capacity of the heater in order to maintain a higher operating equilibrium temperature than can be reached by heat interchange. In this event either a small fixed resistance unit in series with the winding or an auto-transformer will accomplish the desired result.

*Flask and Joint*—A somewhat larger ball and socket joint is recommended than that previously used. Instead of a 35/20 joint the larger 35/25 size allows a maximum volume of vapor to flow without flooding the condenser.

### *Operation*

The volume of urine used may now be varied between reasonable limits and almost any of the heavier than water solvents can be

used in the extractor. The hole *C* in the plunger *A* is set at such a point that the sintered glass disk just dips into the urine while the extractor is in operation. Trap *F* prevents vapor from escaping through the hole in the plunger.

It is recommended that the extractor be put in operation before the heater is started, in order to provide convection currents to distribute the heat in the liquid.

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# EFFECTS OF INSULIN UPON THE PRODUCTION OF KETONE BODIES

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(Received for publication, June 23, 1941)

In 1936 we reported the occurrence of severe ketosis "immediately following hypoglycemic shock in insulin-treated diabetics, who for some time prior to the insulin shock were well under control on diets containing 120 to 250 gm. of carbohydrates with commensurate doses of insulin" (1). Since 1935, when we first observed this causal connection between hypoglycemia and ketosis, appreciation of the phenomenon and its proper application in the treatment of diabetic patients in our institute have consistently produced gratifying clinical results (2). In the light of this experience the subject assumed sufficient significance to deserve further study. With regard to Claude Bernard's thesis that pathological processes differ only quantitatively from normal physiological processes, our observations were extended beyond diabetic patients to include normal human subjects and laboratory animals. The results of these studies were briefly reported last year in a preliminary note (3).

## EXPERIMENTAL

*Analytical Technique*—The low concentrations of ketone bodies encountered in normal blood made it essential to employ a sensitive analytical method, capable of reliably registering changes that may be relatively great while small in absolute amount. At the outset we used Hubbard's method (4) as the only micromethod then available that was sensitive and accurate, but later we replaced it by a simpler technique (5). The errors in both methods lie within 10 per cent when the amount of acetone derived from the total ketone bodies contained in the sample under analysis is not less than 0.01 mg.

*Normal Subjects*—Studies on healthy individuals show that insulin, when injected in quantities sufficient to cause a protracted state of hypoglycemia, exerts in succession two opposite effects upon ketonemia. When the blood sugar drops below the postabsorptive level, the amount of the ketone bodies diminishes during the first few hours; if, however, the hypoglycemic state is maintained long enough, the amount of the ketone bodies begins to in-

TABLE I  
*Initial Depression and Subsequent Rise of Ketonemia in Healthy Subjects during Insulin Hypoglycemia*

Subject	Insulin injected	Time after insulin injection	Blood sugar	Ketone bodies	Change in ketonemia relative to initial levels
	<i>units</i>	<i>hrs.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>
M. S., male	12	0	86	1.1	
		3	54	0.8	-27
		5	56	1.6	+45
T. E. W., male	15	0	80	0.6	
		1.5	65	0.6	0
		6	50	1.9	+216
E. S., male	15	0	77	0.4	
		1.5	50	0.4	0
		6	44	0.9	+125
C. S., female	12*	0	84	0.4	
		1.5	60	0.3	-25
		5.5	46	0.8	+100
A. T., "	12*	0	81	0.4	
		1.5	61	0.3	-25
		5.5	49	0.6	+50

\* 8 units of insulin were injected at the start of the experiment, another 4 units 1.5 hours later.

crease and, in time, may rise well above the level shown before the insulin injection.

The phenomenon is illustrated in Table I, which contains the results of experiments on five subjects. It may be noted that the first phase of the process does not appear in an emphatic form, but that in the second phase the ketonemic level increases considerably in every case, in one instance to the double, in another to more than the 3-fold of the postabsorptive level.

It was considered probable that prolongation of the hypoglycemic state beyond the 5.5 and 6 hour periods, used in these experiments, would cause further increase in ketonemia; reluctant to impose this condition on human subjects, we used two dogs in experiments in which the hypoglycemic state was extended to 10 hours.

TABLE II

*Changes in Ketonemia during Insulin Hypoglycemia in Normal Dogs*

Each dog received 1 unit of crystalline and 1 unit of protamine insulin per kilo of body weight.

Experiment No.	Time after insulin injection	Dog 2		Dog 3	
		Blood sugar	Blood ketone	Blood sugar	Blood ketone
	hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
I. 16 hrs. after last feeding	0	69	0.6	87	0.4
	1.5	33	0.4	32	0.3
	3	51	0.3	49	0.4
	5	60	0.6	32	0.7
	7	31	3.1	23	2.8
	10	34	4.6	37	2.6
II. Repetition of Experiment I, 3 wks. later	0	74	0.9	85	0.6
	1.5	33	0.8	18	0.5
	3	54	0.9	43	0.5
	5	72	0.7	66	0.4
	7	54	1.4	27	1.7
	10	35	3.5	21	4.2
III. Saline substituted for insulin	0	82	0.6	88	0.4
	5	86	1.1	86	0.5
	10	95	0.4	79	0.3

*Normal Dogs*—Food was withheld from the animals for 14 hours before the experiment. After a blood sample was obtained, 2 units of insulin per kilo of body weight were injected, 1 unit of crystalline insulin intravenously and 1 unit of protamine insulin subcutaneously, the former to produce hypoglycemia as rapidly as possible, the latter for the extension of the hypoglycemic state over the prolonged periods of time desired. For 10 hours following the injection, blood samples were periodically drawn for the estimation of sugar and ketone bodies.

The results, given in Table II, show that the ketone bodies in

the blood just begin to rise after the 5th hour following the injection of insulin, the highest ketonemic level appearing after 10 hours of continuous hypoglycemia. The rate of the increase during the last interval suggests that a further rise might have taken place if the hypoglycemic state had been allowed to persist beyond 10 hours. The first phase of insulin effect upon ketonemia, observed in man, is evident also in the dog, showing up after about the same length of insulin action as in man.

To check this finding, 3 weeks later the experiment was repeated with the same dogs under identical conditions. As may be seen, Experiment II, included in Table II, unfolds the same general picture as Experiment I. A further control consisted in the observation of the variability of the sugar and ketone body content of the blood with all experimental conditions the same as in Experiments I and II, except that saline was substituted for insulin. From the results (Experiment III, Table II) it is obvious that the changes which occurred in the preceding two experiments were directly caused by insulin hypoglycemia.

Comparison of Tables I and II permits the inference that ketonemia during hypoglycemia changes much in the same manner in man and dog. This justifies the assumption that extension of the period of observation in man beyond 6 hours would lead to a further substantial rise of the ketonemic level, similar to that occurring in the dog.

*Diabetic Subjects*—Disposition to develop ketosis is one of the main characteristics of the diabetic condition. It is not surprising, then, that the superimposed ketogenic effect of insulin hypoglycemia elicits a much greater response of increased ketonemia in the diabetic than in the normal individual. (In fact, it was the conspicuous ketonuric response to insulin hypoglycemia, observed by us in 1935 in severely diabetic patients, that directed our attention to the problem.)

With a sensitive micromethod available, we studied recently the effect upon ketonemia of clinically unrecognized degrees of insulin hypoglycemia. Disinclined to subject patients to protracted hypoglycemic states for the purpose of experimentation, we made our observations on subjects who received from their attending physicians a type of treatment which, we suspected, might lead to hypoglycemia during postabsorptive periods, mainly during the night,

without coming to the notice of either physician or patient. We have in mind patients receiving substantial single morning doses (or sometimes two doses) of protamine insulin, and also many patients treated with more than 30 units of unmodified insulin. Analysis of blood samples obtained from such patients between midnight and breakfast time actually showed the conditions which

TABLE III

*Increase in Ketonemia Due to Insulin Hypoglycemia in Diabetic Patient*

The patient received 80 gm. of proteins, 50 gm. of fats, 250 gm. of carbohydrates per day, and 30 units of protamine insulin before breakfast. The glucose tolerance test at the foot of the table indicates a mild degree of diabetes. When no hypoglycemia occurred during the night, ketonemia was normal, while 3 nights later, when hypoglycemia occurred, ketosis developed.

Time	Blood sugar	Blood ketone
<i>1940</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Apr. 8-9		
11 p.m.	184	0.9
1 a.m.	195	0.8
3 "	126	0.9
7 "	88	1.0
Apr. 11-12		
10 p.m.	168	0.5
1 a.m.	63	0.6
3 "	58	1.7
7 "	61	6.4

Glucose tolerance				
Blood sugar, mg. per cent, after ingestion of 100 gm. glucose				
0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
163	330	410	325	277

we anticipated: when hypoglycemia (mostly of moderate degrees) set in and had persisted for several hours, the ketonemic level increased appreciably.

Of numerous observations of this kind, one is presented in Table III. This example was selected because by lucky coincidence we intercepted blood samples during a night which passed without hypoglycemia, and 3 days later when hypoglycemia did occur dur-



ing the night. By comparing the records of the two nights, one obtains a clear cut illustration of the causal connection between hypoglycemia and ketosis.

*Diabetic Dogs*—Observations were made during protracted hypoglycemic states on diabetic dogs. The animals were partially (about 90 per cent) depancreatized, and showed but slight glycosuria during the first few weeks following the operation. They were fed exclusively with horse meat (*ad libitum*) and received no insulin injections. Glycosuria gradually increased and in about 2 months after operation reached 4 to 15 gm. per day; at this time

TABLE IV

*Changes in Ketonemia during Insulin Hypoglycemia in Diabetic Dogs*

Dog 4 (weighing 10.5 kilos) received 30 units of crystalline and 10 units of protamine insulin. Dog 7 (weighing 11.5 kilos) received 20 units of crystalline and 10 units of protamine insulin.

Dog 4			Dog 7		
Time after insulin injection	Blood sugar	Blood ketone	Time after insulin injection	Blood sugar	Blood ketone
hrs.	mg. per cent	mg. per cent	hrs.	mg. per cent	mg. per cent
0	308	9.1	0	257	20.3
2.5	65	1.4	3	44	13.3
5.5	45	4.3	5	46	8.5
7.5	48	4.6	7	38	15.0
9.5	31	8.7	10.5	35	24.9
			12	27	30.4

ketonuria also set in. Still the animals maintained their initial body weight and looked normal in every respect. In this condition they were subjected to the experiments. About 14 hours after the last food intake, the dogs were made hypoglycemic for extensive periods of time by the injection of both unmodified and protamine insulin.

In Table IV are presented the blood sugar and ketone body values obtained in two such experiments. Dog 4 was under observation for 9.5 hours after the injection of insulin, about 7.5 hours in hypoglycemia. As may be seen, the two phases in the changes of the ketonemic level, which could be discerned in normal subjects and dogs, are much more conspicuous here; the blood ketones first

dropped from 9.1 to 1.4 mg. per cent, only to increase again to 8.7 mg. per cent as the hypoglycemic state persisted. The rather steep rise during the last 2 hours suggests that this tendency would have continued had the experiment not been interrupted at this point. Extension of the period of observation to 12 hours in the next experiment with Dog 7 proved this to be so. In this instance ketonemia, 12 hours after the injection of insulin (about 10 hours of continued hypoglycemia), has risen well above the level it showed before the injection of insulin.

#### DISCUSSION

The ketogenic action of insulin hypoglycemia was first observed by Collip (6) as early as 1922. He described the occurrence of ketone bodies in the urine of normal rabbits in a condition of hypoglycemia following the administration of insulin. Collip's rather vague explanation of the phenomenon is that the injection of "insulin might by lowering the sugar tension of the blood actually jeopardize the oxidation of glucose in the tissues." Raab (7) in 1928 reported that 3 to 6 hours after the injection of 16 units or more of insulin, normal dogs showed, after a transitory decrease, a rise in ketonemia above the basal level. He interpreted his results on the basis of Minkowski's (and Geelmuyden's) purely speculative theory of glyconeogenesis from fatty acids, a process in which ketone bodies are assumed to appear as intermediary products. Changes in ketosis, according to Raab, simply reflect changes in the rate of glyconeogenesis. Burn and Ling (8) in 1928 found that ketonuria of rats, following frequent insulin injections in the course of 2 or 3 days, increased to about 4-fold of the ketonuria of control animals that were kept on an identical diet. In the insulin-treated rats they also observed protracted hypoglycemic states and a decrease of the liver glycogen to less than 0.1 per cent before the marked rise of ketonuria occurred. Burn and Ling, aware of the causal connection between these findings, regarded the ketogenic effect of insulin as something that "might have been foretold" on the basis of "the previously known facts (a) that insulin reduces liver glycogen, and (b) that the amount of ketonuria is inversely proportional to the amount of liver glycogen."

The interpretation offered by Burn and Ling has found additional support in recent years in the work of numerous investi-

gators. One can now understand changes in the metabolism of ketone bodies, which formerly eluded explanation, on the basis of the well established facts (1) that the ketone bodies which appear in blood and urine originate, as a rule, exclusively in the liver (except, perhaps, in extremely high degrees of ketosis), the amount of the ketone bodies showing an inverse relationship to the glycogen content of the liver, and (2) that whereas the liver utilizes none (or at least no appreciable part) of the ketone bodies which it produces, extrahepatic (in the main, muscle) tissues are capable of consuming substantial amounts.<sup>1</sup> It may be postulated, therefore, that, since insulin is without effect upon the utilization of ketone bodies (16-18), the initial drop and subsequent rise in the ketonemic level during insulin hypoglycemia are parallel to, and contingent upon, corresponding changes in the rate of production of ketone bodies in the liver.

It can scarcely escape attention that the changes in ketonemia observed in our experiments show a conspicuous parallelism to the known changes in hepatic glycogenolysis under the same conditions.<sup>2</sup> The first phase of insulin action tends to increase the glycogen content of the liver by inhibiting hepatic glycogenolysis, while the second phase, which develops after protracted hypoglycemic states, tends to deplete the hepatic glycogen stores by enhancing glycogenolysis. Correspondingly, in line with the thesis of the inverse relationship between the glycogen content of the liver and the rate of ketone formation, the ketonemic level declines in the first and increases in the second phase.

#### SUMMARY

Insulin exerts two opposite effects upon the ketonemic level: for some time after injection it causes a decrease, but after protracted states of hypoglycemia it effects a rise. Both of these insulin effects occur in normal as well as in diabetic men and animals, but appear in the diabetic on a magnified scale.

<sup>1</sup> The discovery of these facts grew out of perfusion studies of liver and other organs by Embden and his collaborators. Subsequently other workers, employing improved analytical methods and a wide variety of experimental approaches, concurred without exception with Embden's conclusions (9-15).

<sup>2</sup> For a discussion and bibliography of the subject the reader is referred to Cori's review ((19) pp. 152-154, 201).

The two phase effect of insulin upon the rate of formation of ketone bodies in the liver closely parallels the effect of insulin upon the rate of hepatic glycogenolysis: in the first phase glycogenolysis is inhibited and, with it, the rate of ketone formation decreases, while after persistence of hypoglycemic states for some time, glycogenolysis is stimulated and, with it, the rate of ketone formation increases.

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# THE ULTRAVIOLET ABSORPTION SPECTRUM OF CRYSTALLINE RIBONUCLEASE

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The ultraviolet absorption spectrum of ribonuclease, recently crystallized by Kunitz (1), has been determined and found to resemble that of other protein enzymes. The sample of crystalline ribonuclease was prepared and kindly furnished by Dr. M. Kunitz. It had been crystallized three times in ammonium sulfate and three times in alcohol. According to our determination by the micro-Kjeldahl method, it had a total nitrogen content of 13.28 per cent (average of 13.50 and 13.05 per cent).

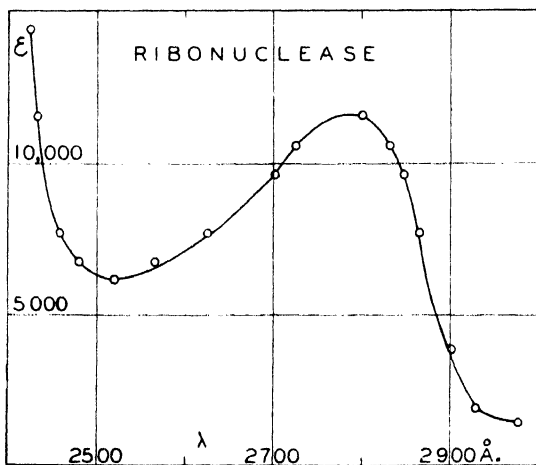


FIG. 1. The ultraviolet absorption spectrum of crystalline ribonuclease

The absorption determinations were made on a solution containing 1.89 mg. of the original crystalline sample per cc. of distilled water. On the basis of a molecular weight of 15,000 and a

nitrogen content of 16.1 per cent for pure ribonuclease (1), this gave a concentration of  $1.04 \times 10^{-4}$  M.

The absorption curve, as measured with a Hilger Spekker photometer and tungsten steel spark source, is shown in Fig. 1. The molecular extinction coefficient,  $\epsilon$ , is defined by the equation  $\epsilon = (1/cd) \log_{10} (I_0/I)$  where  $c$  is the molar concentration and  $d$  is the length of the absorbing solution in cm. A maximum value of  $\epsilon$  of 11,540 occurs near 2800 Å., and a minimum of 6160 occurs at 2520 Å. Almost identical values were obtained for solutions in phosphate buffer at pH 4.5 and in borate buffer at pH 2.5.

In the absence of tryptophane, already shown by Kunitz (1), the wave-length position and magnitude of the absorption maximum are to be ascribed to the tyrosine content (*cf.* (2)). According to our calculations, based on the chemical analysis by Kunitz (1) of the tyrosine equivalent of ribonuclease, there should be twelve tyrosine residues per molecule. Unfortunately there are several conflicting values of  $\epsilon_{\text{max.}}$  reported for tyrosine, but if one accepts Holiday's (2) value of 1240 as the best under the circumstances, then the spectrophotometric evidence indicates nine to ten tyrosine residues per molecule. In view of the several assumptions and sources of error involved, better agreement would not be expected.

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# A PHOTOCHEMICAL YIELD FOR THE INACTIVATION OF CRYSTALLINE TRYPSIN

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That enzymes can be inactivated by exposure to ultraviolet light has been demonstrated qualitatively by a number of investigators (1), but very few experiments have been conducted under conditions which permitted a calculation of the number of molecules inactivated per quantum of radiation absorbed. Obviously data of the latter type can be secured only for pure enzymes whose molecular weight is known. Quantum yields (molecules inactivated per quantum absorbed) as a function of wave-length for the inactivation of crystalline urease have been determined by Landen ((2), *cf.* (3)), and yields for pepsin can be calculated from the data of Gates ((4), *cf.* (2)). The very low yields (around 0.001) obtained for urease and pepsin did not permit any definite conclusions regarding the structure of these two protein molecules or the mechanism of their photochemical inactivation.

With the hope of securing more detailed information under varied experimental conditions, and possibly higher quantum yields, it was considered desirable to extend this type of investigation to other enzymes. Trypsin has been selected for this purpose because it was known to consist of a single molecular species and because several synthetic substrates for it had been prepared by Bergmann and his coworkers (5).

The present paper reports the absorption spectrum of crystalline trypsin as well as quantum yields for its inactivation at  $\lambda$  2537 and 2804 Å.; the yields are based on its loss of ability to release COOH groups in  $\alpha$ -benzoyl-L-arginineamide hydrochloride.



## EXPERIMENTAL

*Absorption Spectra*—As far as the writers are aware, no absorption spectrum for crystalline trypsin has previously been published. From its known protein nature and its content of aromatic amino acids, one would predict its spectrum to resemble that of pepsin. The absorption curve shown in Fig. 1 indicates this to be true, with the maximum value of the molecular extinction coefficient ( $\epsilon_{\text{max.}}$ ) for trypsin occurring at the same wave-length as for pepsin. For trypsin,  $\epsilon_{\text{max.}}$  is equal to 50,000, while the cor-

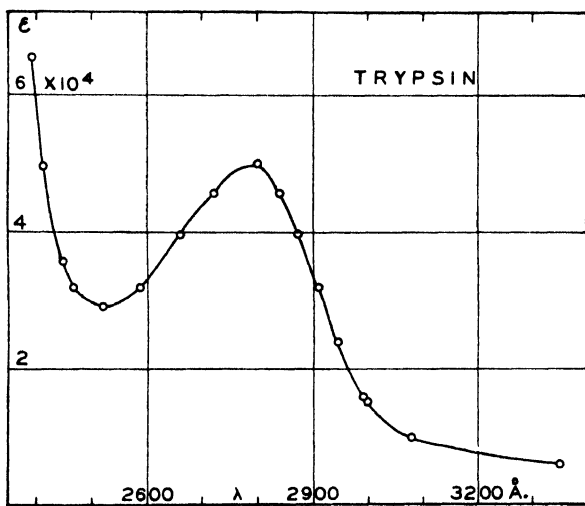


FIG. 1. The ultraviolet absorption spectrum of crystalline trypsin

responding value for pepsin interpolated from the curve of Gates (6) lies in the neighborhood of 60,000. Likewise the minima occur at the same wave-length position, but  $\epsilon_{\text{min.}}$  for trypsin is 29,000, while for pepsin Gates obtained a value between 20,000 and 25,000.

The absorption measurements were made in the University of Missouri Spectrographic Laboratory by Dr. V. R. Ells. A Hilger medium quartz spectrograph and Spekker photometer, together with 1 cm. absorption cells and a tungsten steel spark source, were employed.

The sample of crystalline trypsin, which had been precipitated

with  $\text{MgSO}_4$ , was kindly furnished by Dr. M. Kunitz. For the molecular extinction coefficient, where  $\epsilon = (1/cd) \log_{10} (I_0/I)$ , the molecular weight was taken as 36,500 (7). The concentration is expressed in moles per liter and is based on a determination of the total nitrogen in the sample, with the value of Northrop (8) for the N content (15.0 per cent) of pure crystalline trypsin. The spectrum was obtained with a  $2.52 \times 10^{-5}$  M solution of trypsin in M/15  $\text{KH}_2\text{PO}_4$  buffer (pH 4.5).

An analysis of the crystalline trypsin sample showed that it contained 65.7 per cent trypsin (corresponding to a value of 9.86 per cent found for total nitrogen), 17.0 per cent  $\text{SO}_4$ , 4.4 per cent Mg (spectroscopic), and 0.02 per cent Ca (spectroscopic). The rest was considered to be water of crystallization.

Determinations of absorption spectra for irradiated solutions of trypsin in phosphate buffer revealed only slight increases in the extinction coefficient at the end of 30 minutes irradiation, at which time the activity was reduced to 62 per cent.  $\epsilon_{\text{max}}$  increased 4 per cent and  $\epsilon_{\text{min}}$  6 per cent during this period. This behavior also parallels rather closely the pepsin data of Gates (4), who used the total radiation from a mercury arc; in our case only the wavelength 2537 Å. was employed.

*Activity Determinations*—An approximately  $2.52 \times 10^{-5}$  M trypsin solution was made up fresh for each series of trials in M/15 phosphate buffer at pH 4.5. An 0.8 cc. sample was irradiated, the monochromator being used. The substrate employed with the trypsin was  $\alpha$ -benzoyl-L-arginineamide hydrochloride (5) which was kindly furnished by Dr. Max Bergmann. A 0.0383 M solution of the substrate was made up in M/15 phosphate buffer to which sufficient NaOH was added to give a pH of 11.4. Following irradiation, 0.5 cc. of the exposed trypsin solution was added to 0.5 cc. of the above substrate, giving a resultant pH of 7.3. The mixture was then incubated for  $1\frac{1}{2}$  hours at  $40^\circ$ .

The titration of the carboxyl groups liberated was carried out by a modification of the method due to Grassmann and Heyde (9). It was necessary to make a determination on a blank as well as on the irradiated and control solutions which were incubated. The blank solution was prepared as follows: To 0.5 cc. of unexposed trypsin solution and 0.5 cc. of the same substrate solution were added 1 drop of 0.1 per cent alcoholic thymolphthalein and

just enough 0.2 N NaOH to give a faint blue color. Thus the solution was at once brought to approximately pH 10, which resulted in the loss of tryptic activity.

The titration of the blank was carried out as follows: To 0.2 cc. of the given mixture were added 2 more drops of the same indicator, plus 1.8 cc. of absolute alcohol. The mixture was then titrated with 90 per cent alcoholic 0.01 N KOH until the depth of blue produced was equal to that of a  $\text{Cu}(\text{NH}_3)_4^{++}$  standard (0.0025 M  $\text{CuCl}_2$  + excess  $\text{NH}_4\text{OH}$ ) containing a small amount of  $\text{BaSO}_4$ . The  $\text{BaSO}_4$  was added to give the standard a turbidity comparable to that resulting from precipitation of the trypsin in the titration mixture. The number of cc. of 0.01 N KOH required was taken as the value of the blank.

To a control made up of 0.5 cc. of unexposed trypsin solution and 0.5 cc. of substrate, and incubated for  $1\frac{1}{2}$  hours at  $40^\circ$ , was added the same amount of 0.2 N NaOH as was required by the blank, plus 1 drop of the indicator. The irradiated samples were treated in the same manner. Then 0.2 cc. samples from each were titrated in the same manner as the blank. The fraction of the original trypsin activity remaining after irradiation was found by taking the ratio of the number of cc. of 0.01 N KOH required for the irradiated sample and the control, respectively, after having made a correction for the blank.

*Irradiation*—The source of ultraviolet radiation was a water-cooled capillary mercury arc, operating at atmospheric pressure, in conjunction with a crystal quartz monochromator (*cf.* (2)). The exit slit had an area of 10.08 sq. mm. The rectangular irradiation cell was 4 mm. wide, 40 mm. tall, and had a path length in the direction of the incident radiation of 1.0 cm. At  $\lambda$  2537 Å., 0.744 of the incident energy was absorbed by trypsin molecules at the concentration used; at  $\lambda$  2804 Å., 0.846 of the radiation was absorbed by the trypsin. The volume of the irradiated solution was 0.8 cc.; during treatment, it was stirred continuously with a quartz rod.

The number of ultraviolet quanta absorbed per cc. of solution by active trypsin molecules is given by the product of the following factors: (exposure per unit volume) (average galvanometer deflection) (quanta per erg) (fraction of energy absorbed) (1074) (correction factor). The correction factor takes into account the

decreasing fraction of the total energy which is absorbed by active trypsin as the irradiation treatment progresses. The number 1074 represents the energy incident on the irradiation cell in ergs per sq. cm. per minute per cm. galvanometer deflection.

TABLE I

*Quantum Yield Determinations at  $\lambda$  2537 Å. for Inactivation of Trypsin*

Trypsin concentration	Inactivation	Molecules inactivated per cc. in units of $10^{15}$	Exposure time	Average galvanometer deflection during interval	Correction factor	Yield in molecules per quantum
mg.* per cc.	per cent		min.	cm.		
1.39	29.8	4.52	21	117.6	0.85	0.017
1.43	25.9	4.04	20	114.1	0.86	0.012
1.43	16.0	2.50	20	74.8	0.93	0.014
1.43	27.4	4.27	20	116.1	0.86	0.016
1.43	35.0	5.46	20	110.2	0.81	0.023
1.42	21.8	3.38	20	115.8	0.89	0.013
1.42	36.2	5.61	20	95.5	0.81	0.028
1.42	30.8	4.77	20	77.2	0.84	0.028
1.41	32.2	4.95	20	143.0	0.83	0.016
1.41	24.5	3.68	20	114.5	0.87	0.014
1.41	27.8	4.28	26	74.6	0.85	0.020
1.39	20.0	3.03	20	103.2	0.90	0.012
1.39	22.8	3.46	24	97.5	0.88	0.013
1.39	23.0	3.49	24	90.6	0.88	0.014
1.39	21.5	3.26	24	84.5	0.89	0.014
1.40	29.2	4.46	24	94.4	0.88	0.017
1.40	20.0	3.05	30	70.3	0.90	0.012
1.39	53.5	8.11	26	128.2	0.69	0.027
1.39	45.1	6.84	20	124.8	0.75	0.028
1.39	20.5	3.11	20	115.1	0.90	0.011
Average.....						0.017
						$\pm 0.001$

\* Weight of original sample, uncorrected for true trypsin content (65.7 per cent).

## DISCUSSION

The individual determinations of the number of molecules of trypsin inactivated per quantum of radiation absorbed are tabulated in Tables I and II for the wave-lengths 2537 and 2804 Å., respectively. At 2537 Å. the average of twenty separate deter-

minations gives a value of 0.017; at 2804 Å., the average of fourteen trials is 0.016. Thus no difference in yield with wave-length is indicated in this region.

From the fact that the concentration of active trypsin decreases exponentially with time of irradiation (*cf.* (4)) it is concluded that a molecule of the enzyme must be inactivated by the absorp-

TABLE II

*Quantum Yield Determinations at  $\lambda$  2804 Å. for Inactivation of Trypsin*

Trypsin concentration	Inactivation	Molecules inactivated per cc. in units of $10^{10}$	Exposure time	Average galvanometer deflection during interval	Correction factor	Yield in molecules per quantum
<i>mg.* per cc.</i>	<i>per cent</i>		<i>min.</i>	<i>cm.</i>		
1.39	38.3	5.81	40	72.9	0.79	0.015
1.39	35.3	5.35	40	68.1	0.81	0.015
1.39	32.1	4.87	40	66.6	0.83	0.014
1.39	34.1	5.17	40	64.8	0.82	0.015
1.39	32.0	4.85	24	105.8	0.83	0.014
1.39	30.9	4.68	24	103.5	0.84	0.014
1.39	29.3	4.44	24	104.1	0.85	0.013
1.44	25.3	3.97	24	101.7	0.87	0.012
1.44	35.4	5.56	24	92.4	0.81	0.019
1.40	46.8	7.15	40.2	81.5	0.74	0.019
1.40	37.6	5.74	40	64.9	0.80	0.017
1.40	40.3	6.15	25	100.6	0.78	0.019
1.40	36.2	5.53	30	95.5	0.81	0.015
1.40	46.2	7.05	30	92.6	0.75	0.020
Average .....						0.016 ±0.0005

\* Weight of original sample, uncorrected for true trypsin content (65.7 per cent).

tion of a single ultraviolet quantum. On the average, however, only 1 quantum out of 60 is thus effective. Since these quanta are absorbed by the aromatic amino acid residues, of which there are probably about twenty in the trypsin molecule, the question arises as to whether some particular aromatic residue must absorb a quantum to produce inactivation of the molecule (with a yield of around one-third for that residue) or whether any aromatic group may do so, but with a yield of about one-sixtieth. It is

also conceivable that there may be several regions within a single molecule possessing enzymatic activity. A continuation of this study with other substrates is expected to help answer these questions.

#### SUMMARY

The ultraviolet absorption spectrum of crystalline trypsin has been determined and found to have a maximum ( $\epsilon = 50,000$ ) near 2800 Å., like numerous other proteins.

The number of trypsin molecules inactivated per quantum of radiation absorbed at 2537 and 2804 Å. are 0.017 and 0.016, respectively, where inactivation is defined as the loss of ability to liberate carboxyl groups in  $\alpha$ -benzoyl-L-arginineamide hydrochloride.

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# CHEMICAL STUDIES OF BULL SPERMATOZOA. THE METHIONINE CONTENT OF WHOLE SPERMATOZOA AND OF THE PARTS OBTAINED BY PHYSICAL MEANS\*

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Previous studies (1, 2) with bull sperm have shown that over two-thirds of the 1.6 per cent sulfur present is accounted for as cystine plus cysteine. Studies with the fractions obtained after sonic treatment have given results of the same magnitude. In an attempt to account for the remaining sulfur the determination of the sulfur-containing amino acid, methionine, was carried out by the Baernstein hydriodic acid-hydrolysis procedure (3, 4). The methionine content has been calculated from the methyl iodide liberated and the homocysteine formed. Further, the cystine has been determined iodometrically for comparison with the previously reported values (1, 2). The method has been made more trustworthy by using the observation that hypophosphite, used to prevent oxidation of the hydriodic acid, does not react with iodine at 0° during the cysteine titration.

## EXPERIMENTAL

The Kassell and Brand (5) modification of the Baernstein (3, 4) procedure was used. The apparatus<sup>1</sup> and the procedure were similar to those used by Kassell and Brand. However, we found a

\* This work has been aided by a grant from the National Committee on Maternal Health, Inc.

<sup>1</sup> Loose, glass wool plugs were used in the bottom of the inlet tubes in the train to aid in dispersing the vapors and permit more even regulation of flow. Care was taken to avoid leaks caused by the slight back pressure. Careful grinding of the joint between the digestion flask and the condenser was necessary.



small flame more satisfactory for the heating than an oil bath kept at  $150^{\circ}$ , since low results were obtained with the latter. The flame was adjusted to insure gentle boiling and to maintain refluxing at about the middle of the condenser.<sup>2</sup>

We found that the potassium hypophosphite added to preserve the HI was not always completely destroyed during the hydrolysis. The uptake of iodine by the undestroyed hypophosphite (6) causes high cysteine values. Some workers (4, 5) have concluded that the hypophosphite is completely destroyed; others add only small amounts as needed (7) or evaporate to dryness repeatedly (8) to insure decomposition. Although the hypophosphite is usually destroyed at the temperature obtained with the flame, we have found a simple expedient that prevents the interference of hypophosphite in the cysteine titration and so for this reason its complete destruction is not important. The titration was carried out at  $0^{\circ}$ , a temperature at which we have observed that hypophosphite does not react with the iodine but at which the iodometric titration of the cysteine proceeds smoothly and quantitatively.<sup>3</sup> A separate experiment showed that at  $56^{\circ}$  0.13 gm. of  $\text{KH}_2\text{PO}_2$ , equivalent to 12.5 cc. of a 0.1 M solution, was required to discharge the iodine color from 5 cc. of 0.1 N acid potassium iodate after treatment with excess KI and acidification with  $\text{HCl}$ , whereas at  $29^{\circ}$  2.0 gm. were required, and at  $0^{\circ}$  even a large excess did not discharge the color. It is therefore recommended that the digest be cooled to  $0^{\circ}$  before the iodate is added and kept at this temperature during the titration with thiosulfate.<sup>4</sup>

<sup>2</sup> Once during the subsequent concentration of the digest over an open flame, an explosion occurred which shattered the digestion flask. A shield is recommended for this part of the procedure. Others (6) have noted explosions when a solution of sodium hypophosphite was evaporated on a water bath.

<sup>3</sup> This procedure was used initially because it is known (9) that the conditions (concentration of iodide, excess of iodine, temperature, etc.) for the iodometric titration of cysteine are not as critical when the titration is made at  $0^{\circ}$ .

<sup>4</sup> The homocysteine titrations gave identical values whether made at room temperature or at  $0^{\circ}$ . Presumably the hypophosphite had been destroyed by previous manipulation or did not take part in the reaction under these conditions.

### Results

When the procedure was performed with 3 to 10 mg. samples of methionine,<sup>5</sup> the average recovery of the volatile iodide was 88.5 per cent and of homocysteine was 86.2 per cent. Accordingly, the methionine values in Table I have been corrected by the factors 1.13 and 1.16, respectively. Kassell and Brand (5) who have used factors of 1.07 and 1.12, respectively, have given a discussion of the causes of incomplete recovery. Several determinations of the

TABLE I

*Comparison of Sulfur Data for Bull Spermatozoa and Parts Obtained by Physical Means\**

Fraction	Methionine, volatile iodide†	Methionine, homo- cysteine‡	Cystine, iodo- metric	Cystine§	Total sulfur	Total sulfur ac- counted for
	per cent	per cent	per cent	per cent	per cent	per cent
Whole sperm....	1.92±0.1	1.76±0.1	3.8±0.2	4.1±0.1	1.6±0.1	94
Heads.....	1.01	1.01	4.1	4.0	1.6	81
Midpieces.....	2.25	2.24	4.5	4.4	1.8	94
Tails.....	3.4	4.1	2.4	3.3	1.5	112

\* All samples were made lipid-free and dried by the use of acetone followed by petroleum ether (2). The data are not corrected for the ash content of the samples.

† Corrected by the factor 1.13. Methionine sulfur =  $0.215 \times$  methionine.

‡ Corrected by the factor 1.16.

§ Previously reported data. The cystine values were obtained by using cuprous oxide to reduce and precipitate the cystine; then the sulfur content of the resulting mercaptide was determined gravimetrically or its cystine content by the Sullivan colorimetric method (1, 2). Cystine sulfur =  $0.267 \times$  cystine.

|| Previously reported data. The sample was oxidized in a Parr bomb and the sulfur determined gravimetrically as barium sulfate (2).

methionine and cystine in purified casein (Harris) have given reported values (4, 5). Methionine, added to both casein and whole sperm, has been accounted for quantitatively.

The methionine values, calculated from the volatile iodide and homocysteine, and the iodometric cystine values are summarized

<sup>5</sup> *dl*-Methionine (Hoffmann-La Roche) purified according to Barger and Weichselbaum (10), which gave theoretical sulfur and nitrogen contents.

in Table I, and compared with the cystine and total sulfur values previously determined.

#### DISCUSSION

The cystine<sup>a</sup> values obtained by the Baernstein method (Table I) confirm those previously reported (2) with the exception of the value found for the tails. A comparison of the methionine data calculated from the methyl iodide and the homocysteine shows that in general a good agreement was obtained. The reason for the disagreement between the two methionine values for the tails is not known. It was also found that in the case of the tails, the sum of the average methionine sulfur and cystine sulfur values is greater than the total sulfur content obtained by direct determination. This indicates that the lower of the two methionine values for the tails is the more correct one. The sperm heads contain large amounts of thymus type nucleic acid (2). However, the methylated pyrimidine, thymine, present in this type of nucleic acid, is not likely to be demethylated during the hydrolysis with HI, since the methyl group is bound to carbon. The close agreement of the methyl iodide and the homocysteine values shows that demethylation of the thymine did not occur. Although the sulfur accounted for in the heads is apparently only 81 per cent of the total, the difference is close to the maximum accumulated error of the values used in the calculation and does not necessarily indicate that sulfur compounds, other than those determined, are present. A variation of the cystine sulfur to methionine sulfur ratio from 1.09 in the tails to 4.87 in the heads indicates that more than one sulfur-containing protein is the source of the sulfur.

The hydrolysates of the sperm samples were clear but a trace of humin appeared as a small viscous drop on the surface of the hydrolysate. Lugg (7) had observed humin in HI hydrolysates of plant proteins. The amount of humin was not sufficient for a sulfur analysis; but the loss of sulfur, if any, in this amount of material must have been small.

<sup>a</sup> Cystine and cysteine were not distinguished by the methods used. We have used the word cystine to include both for brevity and because nitroprusside tests had shown the preponderance of cystine over cysteine.

## SUMMARY

The Baernstein method was used to determine methionine and cystine in bull sperm and the fractions obtained after sonic disintegration. 1.92 per cent methionine was found in the whole sperm and a range of 1.01 to 3.8 per cent in the parts. The cystine determinations confirmed previous results obtained by other means. Most of the total sulfur was accounted for by these amino acids. The determination of cystine by the Baernstein method has been made more trustworthy by performing the titration of cysteine at 0°, a temperature at which any undestroyed hypophosphite, used to preserve the HI, was unreactive.

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# GLYCOLYSIS AND PHOSPHORYLATION IN BRAIN EXTRACTS\*

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Geiger (1) reported that cell-free brain extracts, when highly diluted, will form large amounts of lactic acid from added glucose or fructose, but hardly any from hexose monophosphate or hexose diphosphate, and concluded that the above esters are not normal intermediaries of brain glycolysis. His results were the more surprising as glycolysis of the sugars led to accumulation of phosphate esters and required all the coenzymes which are known to be active in so called phosphorylating glycolysis.

Upon repetition of Geiger's experiments it was found that dilute rat brain extracts will form lactic acid from either hexose monophosphate or hexose diphosphate,<sup>1</sup> at the same rate as from glucose or even faster. Per 1 gm. of fresh brain, 30 to 40 mg. of lactic acid are formed in 90 minutes from either glucose or the hexose phosphates.

## EXPERIMENTAL

The extracts were prepared by grinding 1 part of brain with 4 parts of ice-cold distilled water and centrifuging off the solid residue. To 0.2 cc. of the fresh, undialyzed, extract (equivalent to 40 mg. of brain) the following substances were added so that their final concentrations were as indicated: 0.008 M  $Mg^{++}$  (as  $MgCl_2$ ), 0.01 M phosphate buffer, pH 7.3, 0.024 M sodium bicarbonate, 0.0007 M adenosine triphosphate, and 0.0005 M cozymase. After addition of the substrate or substrates, the volume was

\* This work was supported by a research grant from the Rockefeller Foundation.

<sup>1</sup> The esters were prepared in this laboratory by enzymic methods and identified in the usual way. They were used as sodium salts.

brought to 2.0 cc. and the mixtures were incubated at 38° with 95 per cent nitrogen and 5 per cent carbon dioxide in the gas phase. Glycolysis was measured either manometrically or by chemical estimation of lactic acid (2). A typical manometric experiment is illustrated in Fig. 1. The figures are averages of two experiments

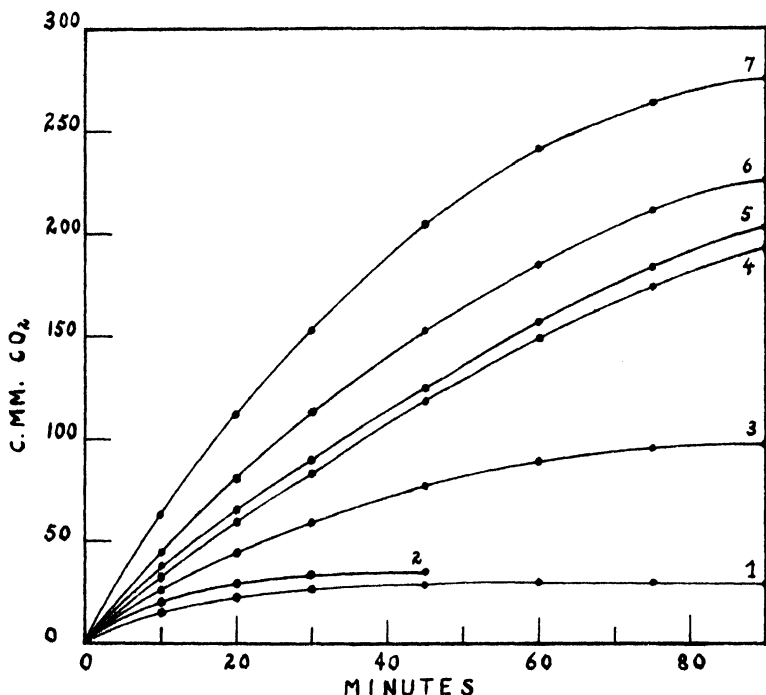
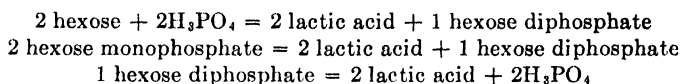


FIG. 1. Glycolysis in rat brain extract; manometric measurement of acid production. Curve 1, no substrate; Curve 2, 0.028 M glucose (no cozymase); Curve 3, 0.014 M glucose; Curve 4, 0.028 M glucose; Curve 5, 0.010 M hexose diphosphate; Curve 6, 0.010 M hexose diphosphate plus 0.030 M creatine; Curve 7, 0.014 M hexose monophosphate.

in which corresponding samples gave results differing by no more than 10 per cent. In the experiment reproduced in Table I lactic acid production was determined both manometrically and chemically. The figures for lactic acid calculated from the manometric data have been corrected for changes in reaction due to formation

or breakdown of hexosephosphoric esters<sup>2</sup> but not for the retention of CO<sub>2</sub> by the enzyme mixtures; so that they are a little lower than the figures secured by chemical estimation; considering this the agreement is fairly satisfactory. The changes in directly determined phosphate (inorganic + phosphocreatine P) indicate that the substrates react according to the following equations.



Both Fig. 1 and Table I show that, although addition of a phosphate acceptor is not indispensable for glycolysis of hexose di-

TABLE I  
*Glycolysis in Rat Brain Extract\**

Substrate	Lactic acid formed		Change in directly determined P  mg.
	Determined manometrically  mg.	Determined chemically  mg.	
None.....	0.15	0.19	
Glucose (0.028 M).....	1.22	1.58	-0.36
Hexose monophosphate (0.010 M) ..	1.17	1.38	+0.03
“ diphosphate (0.017 M) .....	1.15	1.20	+0.35
“ “ (0.017 M) + creatine (0.030 M) .....	1.34	1.54	+0.41

\* Incubated 90 minutes.

phosphate, the rate of lactic acid formation is increased on addition of creatine. Glycolysis does occur without the addition of a phosphate acceptor because one (adenosine diphosphate or adenylic acid) is provided by breakdown of the added adenosine triphosphate through the activity of the adenosine triphosphatase present in the extracts.

Dismutation between triose phosphate (formed from hexose diphosphate) and pyruvic acid also takes place rapidly in dilute brain extracts, as shown in Table II. The experiments were carried out in the presence of sodium fluoride to prevent phospho-

<sup>2</sup> At pH 7, 0.25 equivalent of acid disappears per 1 molecule of phosphate set free from hexose phosphate.



glyceric acid from reacting further. Lactic acid was determined chemically. On addition of a phosphate acceptor, whether creatine or glucose, the reaction is accelerated and inorganic phosphate is esterified with formation of either phosphocreatine or hexose diphosphate. The evidence for this is that, relative to the amount

TABLE II

*Dismutation between Triose Phosphate and Pyruvic Acid in Rat Brain Extract*

Substrates, 0.01 M hexose diphosphate and 0.04 M pyruvic acid. P acceptors, 0.028 M glucose or 0.02 M creatine; 0.04 M NaF; 0.0007 M adenosine triphosphate.

Pyruvic acid was determined by the method of Clift and Cook (3) under correction for the triose phosphate present. The sum of triose phosphate and hexose diphosphate is given approximately by the orthophosphate liberated on hydrolysis in 1.0 N H<sub>2</sub>SO<sub>4</sub> at 100° for 180 minutes; phosphoglyceric acid by the difference between total acid-soluble P and the 180 minute hydrolysis value.

Experiment No.	Incubation period	Phosphate acceptor	Lactic acid formed	Pyruvic acid disappeared	P changes			
					Inorganic	Phosphocreatine	Triose phosphate + hexose diphosphate	Phosphoglyceric acid
	min.		mg.	mg.	mg.	mg.	mg.	mg.
1	70	None	0.69	0.58	0.00		-0.17	+0.17
	70	Creatine	1.06	1.02			-0.30	+0.30
	70	Glucose	1.56	1.50	-0.42		-0.20	+0.62
2	60	None	0.61	0.41	+0.04		-0.17	+0.13
	60	Creatine	1.22	1.24	-0.28†	+0.27	-0.36	+0.37
	135	Glucose*	1.13	0.83	0.00		-0.30	+0.30
	135	Glucose	1.81	1.53	-0.29		-0.24	+0.53

\* No adenosine triphosphate added.

† True inorganic P, precipitated with magnesia mixture.

of triose phosphate plus hexose diphosphate that disappears, more phosphoglyceric acid is formed when glucose is added. The esterification of inorganic phosphate does not occur in the absence of added adenosine triphosphate (Table II, Experiment 2). Demonstration of esterification of glucose in this reaction is of some interest, since Meyerhof (4) was unable to obtain it with rabbit brain extracts although he observed esterification of creatine.

Geiger (1, 5) had found that more concentrated brain extracts had little or no glycolytic activity owing to the presence of a thermolabile inhibitor. This has been confirmed; with 1.0 cc. of extract in a volume of 1.5 to 2.0 cc. the glycolytic activity was negligible in all cases. The necessity of cozymase for glycolysis in dilute undialyzed brain extracts (1) has also been confirmed (*cf.* Fig. 1).

The presence in brain of hexokinase (6), an enzyme which transfers the labile phosphate of adenosine triphosphate to hexoses,

TABLE III  
*Hexokinase in Pigeon Brain Extract*

Experiment No.	Glucose	NaF	Incubation period	P	
				Inorganic	Labile P of adenosine triphosphate*
			<i>min.</i>	<i>mg.</i>	<i>mg.</i>
1	0	0	Initial	0.06	0.24
	0	0	15	0.21	0.09
	+	0	15	0.20	0.06
	0	+	Initial	0.06	0.24
	0	+	15	0.08	0.22
	+	+	15	0.07	0.16
2	0	+	Initial	0.08	0.33
	0	+	15	0.10	0.31
	+	+	15	0.08	0.26

\* Difference between P liberated during 10 minutes hydrolysis in 1.0 N H<sub>2</sub>SO<sub>4</sub> and inorganic P.

has now been demonstrated in a more satisfactory manner than had hitherto been possible (1, 7), by using an acetone powder of brain. Adenosine triphosphatase is partly inactivated by the acetone treatment and can then be completely inhibited with fluoride. The brain was ground with 10 volumes of ice-cold acetone and the mixture was filtered with suction; the brain residue was repeatedly washed with ice-cold acetone and dried *in vacuo* over H<sub>2</sub>SO<sub>4</sub>. 1 part of dry powder was extracted with 20 parts of water and the insoluble material was centrifuged off; 0.3 cc. of the extract was made up to 1.0 cc. with 0.015 M veronal buffer, pH 7.5, 0.004 M magnesium chloride, and 0.004 or 0.005 M adenosine

triphosphate, with or without 0.028 M glucose and 0.04 M sodium fluoride; the mixtures were then incubated in air at 30°. Table III shows that, without fluoride, added adenosine triphosphate is mainly dephosphorylated by adenosine triphosphatase, but that with fluoride it transfers phosphate to glucose. This is indicated by the decrease in phosphate hydrolyzed in 10 minutes by normal acid at 100° (labile phosphate of adenosine triphosphate) with no change in the concentration of inorganic phosphate when fluoride is present.

It has been previously reported (7, 8) that phosphorylation of glucose or of hexose monophosphate to hexose diphosphate in brain dispersions can be "coupled" with oxidation of pyruvic acid. Attempts to demonstrate a coupling with dismutation between triose phosphate and pyruvic acid were unsuccessful because the relatively concentrated brain dispersions failed to catalyze the dismutation to a significant extent.<sup>3</sup> It had also been observed that the brain dispersions oxidized glucose or hexose diphosphate at a much lower rate than pyruvic acid. These results must now be attributed to the presence of Geiger's inhibitor in the enzyme preparations. Evidence for phosphate transfer from phosphoglyceric acid to adenylic acid (via phosphopyruvic acid) in brain extracts has been presented in previous papers (7, 9).

In conclusion it can be said that sugar breakdown in cell-free preparations of brain occurs after phosphorylation to hexose diphosphate by the same reactions as in similar preparations of muscle, retina, and other tissues. Whether free hexoses rather than glycogen are attacked would seem to depend on the relative proportions of hexokinase and phosphorylase (10) present in, or extracted from, the tissues. Recent evidence (11) which indicates the occurrence of phosphorylating glycolysis in brain slices is of interest in this connection.

#### SUMMARY

In the presence of magnesium ions, inorganic phosphate, adenosine triphosphate, and cozymase, dilute rat brain extracts form lactic acid at a high rate from added glucose, hexose monophosphate, or hexose diphosphate. Dismutation between triose phos-

\* Ochoa, unpublished observations.

phate and pyruvic acid is markedly accelerated in the presence of a phosphate acceptor such as creatine or glucose; in this case inorganic phosphate is esterified with formation of either phosphocreatine or hexose diphosphate. In aqueous extracts of dry acetone powders of brain, adenosine triphosphatase is completely inhibited by fluoride and the labile phosphate of adenosine triphosphate is transferred to glucose without liberation of inorganic phosphate (hexokinase reaction). The implications of these facts are discussed.

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## THE VERATRINE ALKALOIDS

### XII. FURTHER STUDIES ON THE OXIDATION OF CEVINE

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In an earlier communication (1) the chromic acid oxidation of cevine was reported and the isolation of a crystalline product described which was called decevinic acid. This product could be isolated from the acid fraction which resulted from the oxidation only after preliminary heating of this fraction, preferably to 180°. A more thorough examination of the products which resulted from the oxidation has since been made and is the subject of this report.

The procedure which offered the most promising results was one in which the acid fraction obtained by continuous extraction of the oxidation reaction mixture with ether was esterified with diazomethane and then fractionated by the use of microfractionating columns. Careful examination of each of the resulting fractions indicated the presence of the methyl esters of at least five different acids.

Fractions 1 and 2 appeared to consist mostly of the dimethyl ester of methylsuccinic acid, contaminated with the ester of succinic acid. The methoxyl content of Fractions 4 and 5 was strikingly lower than that of the others. The analytical results combined with the saponification data as well as analyses of the recovered acid indicated a formulation of  $C_{11}H_{14}O_8$  or  $C_{11}H_{16}O_8$  for the acid. Since the ester was completely saponified within 5 minutes when heated with a slight excess of 0.1 N NaOH at 100°, it appears hardly possible that a tertiary carboxyl group is present. 3 moles of alkali on the basis of this formulation were consumed but the methoxyl determination showed the presence of only two methyl ester groups. Two carboxyl groups and a lactone

group must be present in the acid. The nature of the remaining oxygen atoms cannot be decided until the exact formulation of the acid itself is made definite by later work.

Fractions 7, 8, and 9 almost entirely crystallized. The crystalline material on analysis proved to be the *tetramethyl ester*,  $C_{14}H_{22}O_8$ , of a *hexanetetracarboxylic acid*,  $C_{10}H_{14}O_8$ . The ester melted at  $65-66^\circ$  and was optically active; *viz.*,  $[\alpha]_D = +22^\circ$ . The total yield of this ester from all fractions was about 11 per cent of the theory based on the amount of cevine employed.

The acid recovered on saponification of the ester crystallized from ether and melted at  $170-175^\circ$ . This acid after distillation at 0.2 mm. pressure yielded an optically active *dianhydride*,  $[\alpha]_D = +67^\circ$ , which gave analytical data in agreement with the formulation  $C_{10}H_{10}O_6$ . This anhydride was converted into a *ketomonoanhydride*,  $C_9H_{10}O_4$ , when distilled at a higher temperature and pressure. The latter was also optically active,  $[\alpha]_D = +128^\circ$ .

These observations are sufficient to remove a number of possible structural formulas which might be considered for the hexanetetracarboxylic acid. The fact that it first loses 2 molecules of water to give a dianhydride, then loses  $CO_2$  to give the ketomonoanhydride appears to exclude any formula which contains more than a single carboxyl group on the same carbon atom; *viz.*, a substituted malonic acid. The latter was of course already improbable because of the conditions under which cevine was oxidized; *i.e.*, in 25 per cent sulfuric acid solution at boiling temperature. The carboxyl groups must therefore be distributed on different carbon atoms on a hexane or isohexane chain.

Further information was obtained by a study of the rate of hydrolysis of the tetramethyl ester in alkaline solution. This rate is shown by Curve 1, Fig. 1. In the curve time is plotted against the equivalents of alkali consumed. A weighed quantity of the ester, 30 mg., was placed in the steam bath with 5 equivalents of 0.1046 N NaOH. For withdrawal of the aliquot part (0.1 cc.) at the intervals indicated on the graph, the solution was quickly cooled to  $20^\circ$ , the sample taken, and the solution at once heated again. The aliquot was then titrated against standard acid with phenolphthalein. Although the ester was not completely soluble at first, on stirring it completely dissolved in a few seconds.

The data show that 2 equivalents are so rapidly consumed that

the rate of hydrolysis of two of the ester groups cannot be measured by this technique. In fact, a separate experiment in which the ester was heated with only 2 equivalents of 0.1046 N alkali for a short time revealed complete hydrolysis of these two groups.

Examination of the remainder of the curve reveals that the other two methyl ester groups are much more difficult to saponify and in fact can hardly be saponified with the slight excess (1 equivalent of 0.1046 N NaOH) of alkali used. However, saponification was complete after prolonged heating with excess 1.01 N NaOH.

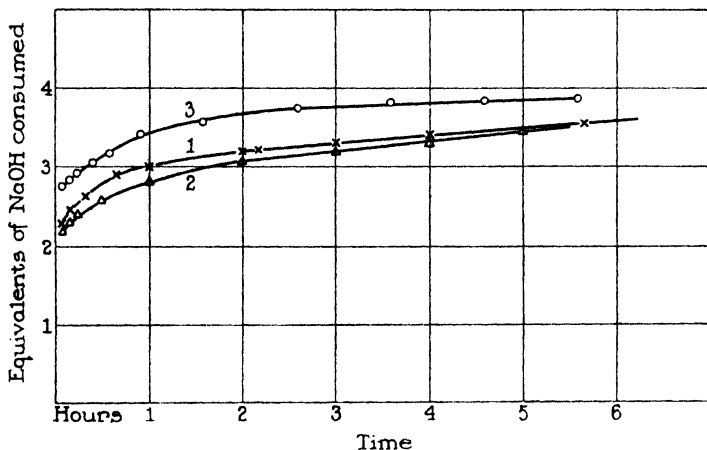


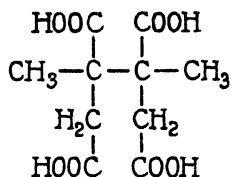
FIG. 1. Hydrolysis of methyl esters in alkaline solution. Curve 1, hexanetetracarboxylic acid methyl ester; Curve 2, heptanetetracarboxylic acid methyl ester; Curve 3, methyl ester of the precursor of decevinic acid.

It can thus be assumed with a fair degree of certainty that two tertiary carboxyl groups must be present, since the difficulty of saponification of the ester of such a carboxyl group (where there is no complication from adjacent double bonds or rings) as contrasted with the ease of saponification of a primary or secondary carboxyl group is well known. The esters from Fractions 2 and 5 as well as the trimethyl ester of tricarballic acid taken as a model were completely hydrolyzed within 5 minutes under analogous conditions.

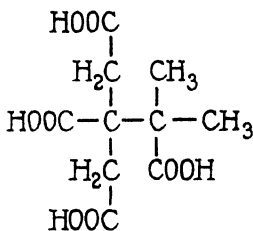
If the hexanetetracarboxylic acid contains two tertiary carboxyl



groups, only two formulas are possible under the limitations set by the formulation  $C_{10}H_{14}O_8$ . These are given in Formulas I and II.



I



II

Formula II contains no carbon chain longer than that of glutaric acid and thus could not directly give rise to the ketomonoanhydride on distillation. Formula I therefore seems to be indicated for our acid, although it cannot be considered certain until synthetic experiments planned in the near future are completed and actual identity established. Synthetic confirmation is made more difficult by the fact that the acid and its derivatives from cevine are optically active.

The carbon content of each of the fractions from Fraction 10 on was higher than that of those which preceded. These fractions did not crystallize. Analysis of Fraction 12 suggested the presence of the *tetramethyl ester*,  $C_{15}H_{24}O_8$ , of the next higher homologue of the above acid; i.e., a *heptanetetracarboxylic acid*,  $C_{11}H_{16}O_8$ . This ester, like that of the hexanetetracarboxylic acid, proved to be difficult to saponify completely and required prolonged heating with normal alkali. The crystalline acid saponification product melted at  $145\text{--}148^\circ$ . The analytical data were in good agreement with those required by the formulation  $C_{11}H_{16}O_8$ . This acid was obtained in much smaller amount from cevine than was the hexanetetracarboxylic acid.

The rate of hydrolysis in 0.1046 N NaOH of this tetramethyl ester was determined as in the previous case. The results obtained are reproduced in Curve 2, Fig. 1. It is apparent that this acid also must contain two tertiary carboxyl groups.

After Fraction 14 the distillates were viscous resins. The

analytical data of Fraction 17 recovered by sublimation were in fair agreement with the formulation  $C_{17}H_{24}O_8$  containing three methoxyl groups as ester groups. A portion of the latter proved to be comparatively difficult to saponify. Since 4 equivalents of alkali were consumed and only three methyl ester groups can be present, the extra equivalent of alkali must have been required for cleavage of a lactone group. The lactone acid would therefore possess the formulation  $C_{14}H_{18}O_8$ .

The rate of saponification of the ester which was determined as in the case of the hexanetetracarboxylic acid is reproduced in Curve 3, Fig. 1. From this curve it can be seen that there must be present the ester of at least one tertiary carboxyl group and of another which is less readily hydrolyzed than the other two and possibly also of tertiary character.

The acid which was recovered from the saponification mixture failed to crystallize. This acid, however, proved to be the precursor of decevinic acid. On pyrolysis, only water and no  $CO_2$  was liberated.<sup>1</sup> The yield of recrystallized decevinic acid obtained was 78 per cent of the theory on the basis of the formula  $C_{14}H_{18}O_8$  for the acid. This high yield of decevinic acid,  $C_{14}H_{14}O_6$ , strongly supports the conclusion that the above acid is its precursor with the above formulation and that of its ester is  $C_{17}H_{24}O_8$ .

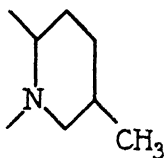
The transition from this acid to decevinic acid would appear superficially to be rather simple and to be brought about by the loss of only 2 molecules of water. However, the former is obtained by strong alkaline hydrolysis, whereas decevinic acid is extremely sensitive to alkali and loses 2 molecules of  $CO_2$  under such treatment. In the precursor all of the oxygen atoms are accounted for in the three carboxyl groups and the lactone group and a rearrangement must occur during the heating, so that at least one of the carboxyl groups is transformed into some other grouping in the production of decevinic acid. Even after prolonged heating of the latter in strong alkali, only 3 equivalents of alkali are consumed and a ketonic group is then found to be present in the crystalline hydrolytic product,  $C_{12}H_{16}O_3$  (1).

The empirical formula  $C_{14}H_{18}O_8$  for the precursor of decevinic

<sup>1</sup> It was originally incorrectly assumed that decevinic acid was formed by the loss of water and  $CO_2$  during the heating of the crude oxidation product.

acid would allow for the presence of six double bonds or rings. Three of these, however, are accounted for in the three carboxyl groups and two in the lactone group. It seems probable, therefore, that the substance contains a single hydrocarbon ring or, less probably, an open chain with a single double bond. In any case, it is difficult to see how such a substance could possibly give rise to a substance with either of the structural formulas previously discussed by us (1). The structural formula of decevinic acid is therefore still obscure. The production from it of hydroxynaphthalic anhydride described in our earlier work cannot now be taken as evidence for the presence of a hydrogenated naphthalene ring system in cevine. Its formation can be the result of pyrolytic rearrangement.

Careful examination of the original oxidation mixture from cevine has revealed the presence in it of two substances which possess the formulas  $C_6H_9ON$  and  $C_6H_{11}ON$ . Their properties suggest a lactam character. If these substances should arise from the oxidation of that portion of the structure (2) previously discussed, *viz.* Formula III, they could be interpreted as 4-methyl-2-

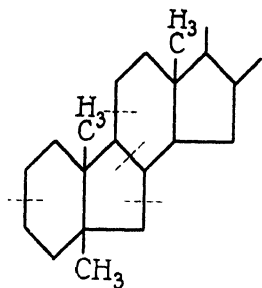


III

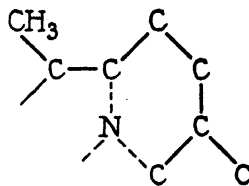
pyrrolidone and 5-methyl-2-piperidone respectively. An attempt will be made in the future to obtain these substances for comparison in order to recheck data already given in the literature.

Finally, there remains to be discussed the bearing that these substances may have on the interpretation of the structure of cevine. An earlier contribution has suggested a possible cyclopentenofluorene structure (3) for the hydrocarbons resulting from the selenium dehydrogenation of cevine and this has been more recently supported by the substances isolated from the dehydrogenation of jervine (4). Thus a suggestion is obtained that at least a portion of the molecule of cevine itself may contain a fully hydrogenated ring system of this type, which, however, will have

to be substantiated by other methods. If for the present such a ring system is assumed, it must be so modified as to permit the production from it of the hexanetetracarboxylic acid. The basic portion of the molecule (2) consisting possibly of an octahydro-pyridocoline or octahydropyrrocoline could hardly give rise to such a structure by oxidative degradation. The structure indicated for the hexanetetracarboxylic acid suggests the presence of two geminal methyl groups on adjoining carbon atoms and could have its origin, barring rearrangement, in the structure shown in Formula IV by oxidative rupture at the points indicated. This formula would also permit the formation of the homologous acid,  $C_{11}H_{16}O_8$ .



IV



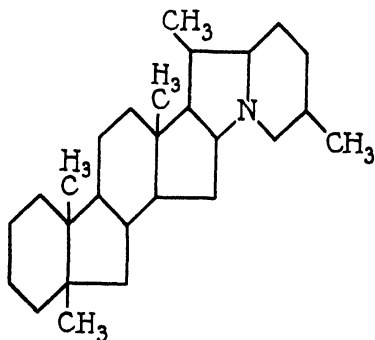
V

Further relationships for the basic portion of the molecule might be suggested on the basis of certain arrangements known to exist in nature. For example, the 2-ethyl-5-methylpiperidine (pyridine) (2) which has so constantly been encountered in our degradative work with cevine might be considered to have its origin in a grouping suggestive of the isooctyl side chain of the sterols as shown in Formula V.

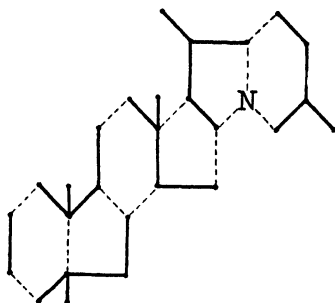
This would resemble somewhat the condition which has been found to exist in the study of the *Solanum* alkaloids which are sterol derivatives and in which the basic portion consists of a dicyclic tertiary base presumably molded from the sterol side chain. The extra benzene ring of the benzofluorene hydrocarbons from jervine if not already present in the molecule may have been formed during the dehydrogenation. In the latter case, ring closure must have taken a different direction (*i.e.*, as in

methylcholanthrene) than it does in the formation of the various 2',1'-naphtha-1,2-fluorenes during the dehydrogenation of the sterols.

Such considerations, if correct, might suggest a ring system for cevine as is given in Formula VI. This ring system might have its origin, although not in the conventional manner, in the condensation of isoprene units and nitrogen with 2 carbon atoms from another source, as shown in Formula VII. Such an interpretation



VI



VII

is suggested with due reservation, since much more in the form of pertinent data remains to be accumulated.

#### EXPERIMENTAL

10 gm. of recrystallized cevine were oxidized as previously reported (1). The ether extract, which in the earlier work was evaporated to an oily residue and which when heated yielded decevinic acid, was only partly concentrated. An excess of diazomethane in ether was then added and, after a few minutes at room temperature, the solution was concentrated. The oily residue weighed approximately 5 gm. A preliminary fractionation of this oil was made in a sublimation apparatus at low pressure. Six fractions were collected. The first five were collected under a pressure of 0.2 mm. and up to a bath temperature of 148°. The last fraction weighed 1.12 gm. and was obtained at 0.001 mm. and 120°.

A preliminary smaller run, on which an accurate fractionation

with the use of a microfractionating column had been accomplished, yielded crystalline material which proved to be the *tetramethyl ester of a hexanetetra-carboxylic acid*. When seeded with this material, Fractions 2, 3, and 4 of the larger experiment crystallized without further fractionation and a total of 0.5 gm. was collected from ether. This substance will be described later.

TABLE I  
*Fractionation of Esters*

Fraction No.	Bath temperature	Column temperature	Pressure	Weight (approximate)	Physical appearance	Micro b.p.	Analysis		
							C	H	OCH <sub>3</sub>
	°C.	°C.	mm.	mg.		°C.	per cent	per cent	per cent
1	130	60	0.5	80	Oil	188	51.27	7.18	
2	155	60	0.5	80	"	199	51.76	7.23	36.84
3	165	110	0.2	80	"	250*	52.01	6.88	31.58
4	170	110	0.2	100	Crystalline	245*	51.72	6.21	21.51
5	180	120	0.2	70	"	250*	51.48	6.04	20.99
6	180	140	0.2	100	Oil		52.11	6.75	36.38
7	187	150	0.2	100	Crystalline		52.64	6.67	
8	190	155	0.2	100	"		53.31	6.91	37.68
9	195	160	0.2	170	"		53.16	7.00	
10	200	160	0.2	170	"		53.70	6.94	37.32
11	200	165	0.2	170	Oil		53.80	7.15	
12	200	165	0.2	170	"		54.03	7.31	36.96
13	210	170	0.2	170	"		53.95	7.00	
14	215	190	0.2	170	Viscous		54.15	6.42	29.90
15	220	195	0.2	70	"		55.64	6.17	
16	220	195	0.2	200	"		56.12	6.62	26.65

\* These boiling points are not reliable; possibly MeOH split off.

The mother liquors from this substance were combined with Fractions 1 and 5. This material was placed in a 22 cm. column (5) and fractionated according to the data given in Table I, which includes also the analytical results.

*Methylsuccinic Acid* (?)—Fraction 2 gave analytical results and a boiling point suggestive of the dimethyl ester of methylsuccinic acid.

C<sub>7</sub>H<sub>12</sub>O<sub>4</sub>. Calculated. C 52.47, H 7.56, OCH<sub>3</sub> 38.75  
Found. " 51.76, " 7.23, " 36.84

69.6 mg. of the ester were treated with 12.46 cc. (3 equivalents) of 0.1046 N NaOH and the mixture was heated at 100°. In 5 minutes 0.1 cc. of this solution was withdrawn and titrated with 0.1046 N HCl against phenolphthalein. 0.0348 cc. of acid was required. This corresponds to 1.96 moles of alkali consumed on the basis of the above formulation. On further heating no additional alkali was consumed.

The saponification mixture after concentration to a small volume was acidified with HCl and exhaustively extracted with ether. The dried extract yielded a residue of 60 mg. 7 mg. of this crystallized from ether. The melting point was not sharp. The substance began to melt at 150° but the crystalline form appeared to change to needles which melted entirely at 185°. It is possible that the material was a mixture of succinic acid and a homologue.

$C_4H_6O_4$ .	Calculated.	C 40.66, H 5.12
$C_6H_8O_4$ .	"	" 45.42, " 6.10
	Found.	" 41.90, " 5.50

Since the mother liquor did not crystallize satisfactorily, the residue obtained by concentration was placed in a sublimation apparatus under 0.2 mm. pressure and allowed to sublime at a bath temperature of 100°. The sublimate crystallized on standing a short time and weighed 30 mg. The melting point of 65–100° indicated a mixture, possibly of methylsuccinic acid with homologues.

$C_8H_8O_4$ . Calculated, C 45.42, H 6.10; found, C 46.03, H 6.54

*The Acid  $C_{11}H_{14}O_8$  or  $C_{11}H_{16}O_8$  (?)*—Fractions 4 and 5 differed from the others by the much lower methoxyl content. If two methoxyl groups are present in the form of a dimethyl ester, the following formulations might be considered in connection with the analytical results.

$C_{13}H_{20}O_8$ .	Calculated.	C 51.29, H 6.62, $OCH_3$ 20.38
$C_{13}H_{18}O_8$ .	"	" 51.63, " 6.00, " 20.52
	Found.	" 51.48, " 6.04, " 20.99

14 mg. of the dimethyl ester were treated with 2.215 cc. of 0.1046 N NaOH (5 equivalents on the basis of the above formulas) and heated in the steam bath. At the end of 5 minutes 0.1 cc. was withdrawn and titrated with standard HCl against phenol-

phthalein. 0.0373 cc. of 0.1046 N HCl was required. This corresponds to a consumption of 3.13 moles of alkali. Further heating did not change the result.

If either of the above formulations is assumed, then there can be no tertiary carboxyl groups present, because of the speed of saponification. The presence of a lactone grouping is indicated as well as that of two methyl ester groups.

70 mg. of the ester were saponified with excess alkali and the mixture was concentrated to a small volume. Excess HCl was added and the solution was exhaustively extracted with ether. This yielded a residue of 60 mg. Although this could not be crystallized from any solvent, it was distilled with the thought that an anhydride might be formed which would crystallize. The temperature of the oil bath was held at 200° and the pressure was then reduced until distillation occurred. Since the distillate did not crystallize, it was analyzed directly.

$C_{11}H_{16}O_8$ .	Calculated.	C 47.80, H 5.84
$C_{11}H_{14}O_8$ .	"	" 48.16, " 5.14
	Found.	" 48.25, " 5.88

Apparently under these conditions the lactone group present in the original ester is reformed but there is no tendency to form an anhydride. 2 of the oxygen atoms in this ester are thus unaccounted for.

*The Hexanetetracarboxylic Acid,  $C_{10}H_{14}O_8$* —Fraction 7 crystallized almost entirely and gave analytical data in fair agreement with those of the crystalline ester obtained from the preliminary experiment mentioned above. Upon recrystallization from ether, 65 mg. of heavy rhombs were obtained which melted at 63–66°. After another recrystallization, the ester melted at 65–66°.

	$[\alpha]_D^{25} = +22^\circ$ ( $c = 0.916$ in methyl alcohol)
$C_{14}H_{22}O_8$ .	Calculated. C 52.80, H 6.97, $OCH_3$ 39.07, mol. wt. 318.18
	Found. " 53.09, " 6.79
	" 52.88, " 6.76, $OCH_3$ 38.12, mol. wt. 297.8

For complete saponification, prolonged heating on the steam bath with excess normal alkali was necessary because of the resistant ester groups. 75.5 mg. of the ester were heated with 1.81 cc. of 1.01 N NaOH at 100° for 4.5 hours and titrated back



with 0.992 N HCl against phenolphthalein. Found, 0.930 cc. of alkali; calculated for 4 equivalents, 0.940.

The neutral solution from the titration was concentrated to a small volume and a slight excess of concentrated HCl was added. It was then exhaustively extracted with ether. On concentration, the residue crystallized. After recrystallization from ether, the acid melted at 170–175°.

$C_{10}H_{14}O_8$ . Calculated, C 45.78, H 5.38; found, C 45.73, H 5.48

*The Dianhydride of the Hexanetetra-carboxylic Acid,  $C_{10}H_{10}O_6$* —Approximately 150 mg. of the acid were placed in a distillation apparatus under 0.2 mm. pressure. Nothing appreciable distilled until the oil bath temperature reached 230°, when a stormy distillation occurred. The distillate partially crystallized. After recrystallization from acetone, 30 mg. of stout, well formed rods were obtained which melted with effervescence at 154–160°, depending somewhat on the rate of heating.

$[\alpha]_D^{25} = +67^\circ$  ( $c = 0.86$  in acetone)

$C_{10}H_{10}O_6$ . Calculated, C 53.20, H 4.47; found, C 52.90, H 4.53

*The Keto Monoanhydride,  $C_9H_{10}O_4$ , from the Hexanetetra-carboxylic Acid*—The mother liquors from the crystallization of the above anhydride were recombined with part of the crystalline material and again placed in the distillation apparatus. The pressure was now maintained at 10 mm., while the oil bath temperature was held at 250°. The distillate weighed 85 mg. and crystallized readily from a mixture of acetone and ether. After recrystallization it melted at 115–118°.

$[\alpha]_D^{25} = +128^\circ$  ( $c = 0.75$  in acetone)

$C_9H_{10}O_4$ . Calculated, C 59.31, H 5.53; found, C 58.95, H 5.49

*The Heptanetetra-carboxylic Acid*—Fraction 12 did not crystallize directly but gave analytical results in agreement with the figures for the ester of an acid containing one  $CH_2$  group more than that of the previous acid.

$C_{11}H_{24}O_8$ . Calculated. C 54.18, H 7.28,  $OCH_3$  37.33

Found. " 54.03, " 7.31, " 36.95

This ester like the previous one also contained two relatively resistant ester groups. 125 mg. of the oil were treated with 5 cc.

of 1.01 N NaOH and heated on the steam bath for 6 hours. The mixture was titrated back against phenolphthalein with HCl. Found, 1.62 cc.; calculated for 4 equivalents, 1.49 cc.

The neutral solution from the titration was concentrated to a small volume and made acid with HCl. It was exhaustively extracted with ether. This yielded on concentration a resin which soon began to crystallize. The crystalline acid after collection with ether melted at 145–148°.

$C_{11}H_{16}O_8$ . Calculated, C 47.80, H 5.84; found, C 48.13, H 5.77

Following Fraction 16 approximately 0.8 gm. of material remained undistilled. It was very viscous and difficult to manipulate in the type of distilling apparatus used. Accordingly, it was removed with solvent and then redistilled in a sublimation apparatus. The colorless viscous distillate will be considered as Fraction 17. The analytical data from this fraction did not differ greatly from those of Fraction 16 and the properties were similar. If it should be assumed to approach homogeneity, the analytical data would agree best with the formulation  $C_{17}H_{24}O_8$  containing three methoxyl groups.

$C_{17}H_{24}O_8$ . Calculated. C 57.27, H 6.79,  $OCH_3$  26.11  
Found. " 56.79, " 6.56, " 26.14

150 mg. of the viscous substance were treated with 4.00 cc. of 1.01 N NaOH and heated in the steam bath for 4.5 hours. On back titration against phenolphthalein with HCl, 1.68 cc. of alkali were found to have been consumed. Calculated for 4 equivalents, 1.67 cc. Apparently a lactone group must be present, since only three methoxyl groups were shown by the above analysis.

The neutral titration mixture was then concentrated to a small volume and made slightly acid with concentrated HCl and exhaustively extracted with ether. The dried ether extract on evaporation to dryness yielded 115 mg. of a resin which did not crystallize.

This resin was heated in an apparatus connected with a series of nitrometers for measuring any evolved gas. The heating was performed in a slow stream of nitrogen at 200° for 10 minutes. The slightly colored residue appeared to be entirely crystalline. After nitrogen was passed through the apparatus, no diminution

in volume could be detected after extraction with alkali.  $\text{CO}_2$  therefore was not liberated during the heating. A small amount of water had condensed on the cooler part of the apparatus.

The crystalline residue was recrystallized from acetone. A total of 79 mg. was obtained which melted at  $275\text{--}278^\circ$ , depending on the rate of heating. The analytical figures agreed with the figures for decevinic acid.

$\text{C}_{14}\text{H}_{14}\text{O}_8$ . Calculated, C 60.41, H 5.20; found, C 60.68, H 5.29

If the formula of the acid on the basis of the analytical data of the trimethyl ester be assumed to be  $\text{C}_{14}\text{H}_{18}\text{O}_8$  and decevinic acid is derived from it by the loss of 2 molecules of water, then the yield of decevinic acid was 78 per cent of the theoretical.

TABLE II  
*Fractionation of Nitrogen-Containing Fraction*

Fraction No.	Bath temperature	Column temperature	Pressure	Weight of fraction	Micro m.p.	Analysis	
						C	H
	$^\circ\text{C.}$	$^\circ\text{C.}$	mm.	mg.	$^\circ\text{C.}$	per cent	per cent
1	117	80	0.8	70	Semiliquid		
2	117	80	0.8	255	55-59	60.44	9.05
3	110	73	0.25	165	53-59		
4	110	73	0.25	165	51-58		
5	113	73	0.25	145	51-59	60.58	9.00
6	115	73	0.25	145	48-58		
7	125	82	0.25	150	Mostly liquid		
8	130	95	0.25	200	35	63.03	9.53
9	140	100	0.25	200	36		

*Basic Fraction from Oxidation of Cevine*—The original acid solution from the oxidation of 40 gm. of cevine which remained after the continuous extraction with ether employed to remove the acid products discussed above was treated with 8 liters of ethyl alcohol. 30 per cent NaOH solution was added until the solution was distinctly alkaline to litmus.  $\text{CO}_2$  was then passed through to neutralize any excess NaOH. The salts were filtered off and washed with alcohol. The filtrate was concentrated to dryness and extracted with 95 per cent alcohol. The extract from this after concentration gave a residue which was suspended in 150 cc. of absolute methyl alcohol and saturated with dry HCl gas at  $0^\circ$ . After the mixture had stood for several hours, the salts were

filtered off and washed with methyl alcohol saturated with HCl. Both crops of salts contained no appreciable organic matter.

The filtrate was again subjected to a repetition of this process. The dried residue was decomposed at  $0^{\circ}$  with 24 per cent NaOH and a small volume of ether. Powdered KOH was then added and after the mixture had been quickly centrifuged the ether layer was drawn off. The ether extraction was twice repeated and the ether extracts were dried over  $K_2CO_3$ . After concentration the residue was fractionated in a 22 cm. column, as given in Table II.

The above procedure was employed with the idea that an amphoteric amino acid fraction might be encountered which could possibly be fractionated as methyl esters. However, with Fractions 2 and 9, shown in Table II, the methoxyl as well as the N-methyl determinations were negative. From the boiling points and analytical data they appear to fit best in the category of pyrrolidones or piperidones.

Fractions 2 to 6 appeared to be nearly homogeneous and gave good analytical data for a methylpyrrolidone or for piperidone. The properties, however, definitely exclude the latter.

$C_6H_9ON$ .	Calculated.	C 60.56, H 9.15, N 13.81
	Found.	" 60.44, " 9.04, " 13.36
	"	" 60.58, " 9.00

After two recrystallizations from ether this material melted at  $58^{\circ}$ .

Fraction 9 gave analytical data approaching the figures required for a substance with the empirical formula  $C_6H_{11}ON$ .

$C_6H_{11}ON$ .	Calculated.	C 63.66, H 9.78, N 12.48
	Found.	" 63.03, " 9.53

After recrystallization from an equal volume of ether, compact rhombs were obtained which melted at  $34-37^{\circ}$ .

Found.	C 63.28, H 9.69, N 12.52
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# THE PHOTOCHEMICAL ABSORPTION SPECTRA OF THE PASTEUR ENZYME AND THE RESPIRATORY FERMENT IN YEAST\*

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The power of monochromatic light to reverse the carbon monoxide inhibition of iron-catalyzed reactions taking place in living cells has been utilized to determine the absorption spectrum of the CO compound of the respiratory ferment in *Torula utilis* and *Bacterium pasteurianum* (1, 2) and more recently that of the Pasteur enzyme in retina (3). Accounting for the largest part of the oxygen consumption of the cell, the *respiratory ferment*, or *cytochrome oxidase* with which it is now generally identified, enables molecular oxygen to accept electrons coming from the cellular substrates via cytochrome. The *Pasteur enzyme* which catalyzes the aerobic inhibition of fermentation also reacts with molecular oxygen, but only small amounts of oxygen appear to be necessary to maintain it in the oxidized state (3, 4).

In order to study the relationship between the Pasteur enzyme and the respiratory ferment in the *same* cell, a system must be selected of which the respiration as well as the aerobic fermentation is sensitive to CO. Retina cannot be used, for CO even in high concentrations does not affect the respiration but only inhibits the Pasteur reaction of this tissue (4, 3). Bakers' yeast was chosen for the present experiments because its respiration is decreased and its aerobic fermentation is increased by CO, and

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these effects of CO are partially reversed by illumination (5). The vigorous respiration and the pronounced Pasteur effect exhibited by these cells at low temperatures (2°) are further advantages for photochemical experiments.

### EXPERIMENTAL

#### *Apparatus and Procedure*

Bakers' yeast,<sup>1</sup> fresh samples of which were supplied at weekly intervals, is washed twice with M/15  $\text{KH}_2\text{PO}_4$ , and then about 200 c.mm. of cells are made up to 2.0 cc. with M/15  $\text{KH}_2\text{PO}_4$  containing 1 per cent glucose. The compensation vessel of the differential manometer contains 2 cc. of the glucose-phosphate solution. Only when 30 to 40 minutes have elapsed after the passage of the gas mixture is the first experimental reading taken. The same batch of yeast is used in comparing the efficiency of a particular wavelength in relieving the CO inhibition of respiration and of the Pasteur effect. The temperature of the bath is 2°.

Fermentation is measured in a cylindrical vessel of 20.2 cc. volume, so that the ratio of gas volume to fluid volume is 10. Under these conditions the respiratory exchange produces no appreciable pressure differences, and the positive pressures result essentially from fermentative  $\text{CO}_2$ . Respiration is measured in 23.1 cc. vessels in the usual way by rapidly absorbing the  $\text{CO}_2$  formed, and recording the negative pressures resulting from the  $\text{O}_2$  consumption. The removal of the KOH from an inner well to the top part of the vessel renders that part of the vessel which contains the yeast suspension and which is exposed to the light identical with the vessel used to measure fermentation. The shape of the vessels in conjunction with the rotating method of shaking presents only a thin layer of cells to the light. An under water mirror allows additional light to enter the vessels (Fig. 1).

The details of the photochemical apparatus and the isolation of the monochromatic radiations have been described previously (3).

*Influence of CO on Metabolism of Bakers' Yeast*—When the  $\text{N}_2$  in an atmosphere also containing  $\text{O}_2$  is replaced by CO, two striking changes take place in the metabolism of the yeast: its respira-

<sup>1</sup> The yeast was supplied by the Federal Yeast Corporation through the kindness of Mr. Sidney Kahn.

tion falls and its fermentation increases (Table I). However, the inhibition of the Pasteur reaction is generally more pronounced than the inhibition of respiration. In the absence of  $O_2$ , CO has no effect on the fermentation. These findings confirm those first reported by Warburg (5).

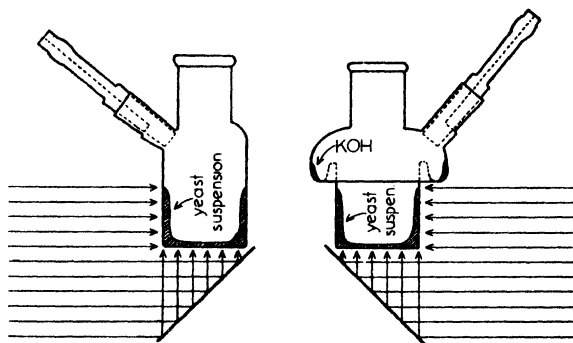


FIG. 1. Manometer vessels (according to Warburg) for measuring fermentation and respiration respectively in the photochemical apparatus. The rotating method of shaking distributes the yeast suspension as a thin film about the walls of the vessel. The arrows indicate the passage of light to the side and bottom of the vessel.

TABLE I  
*Inhibition of Pasteur Enzyme and Respiratory Ferment by CO at 2°*

Atmosphere	$Q_{CO_2}$ (fermentative)	$Q_{O_2}$
100% $O_2$ .....	0- 2	8-10
5% $O_2$ , 95% $N_2$ .....	0- 2	8-10
5% " 95% CO.....	10-12	2- 4
100% $N_2$ .....	13-15	0
100% CO.....	13-15	0

*Determination of Relative Absorption Coefficients ( $\beta_\lambda/\beta_{436}$ )*—The theory underlying the method of measuring photochemical absorption spectra has been developed by Warburg and his collaborators (1, 2, 6, 7). When the intensities of two wave-lengths of light which produce the same photochemical effect are known, the relative light absorption coefficient ( $\beta_1/\beta_2$ ) may be calculated from the following equation,

$$\beta_1/\beta_2 = i_2\lambda_2/i_1\lambda_1$$



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where  $\beta_1$  = absorption coefficient at wave-length  $\lambda_1$ ,  $i_1$  = intensity at  $\lambda_1$ ; and  $\beta_2$  = absorption coefficient at wave-length  $\lambda_2$ , and  $i_2$  = intensity at  $\lambda_2$ .

When  $436\text{ m}\mu$  is the standard wave-length,

$$\beta_{\lambda}/\beta_{436} = i_{436} \times 436/i_{\lambda} \times \lambda$$

For the present experiments the photochemical effects on the CO-treated yeast were in the one instance a decrease in fermentation and in the other an increase in respiration. With the former as the photochemical reaction, points were obtained on the spectrum of the Pasteur enzyme, and with the latter on the spectrum of the respiratory ferment.

It is also necessary to make allowance for the changes in the fermentative and in the respiratory rate which occur with time independently of the illumination. This is done by determining the rate of fermentation and of respiration respectively, before and after each light period. By interpolation it is then possible to determine what the metabolic rate would have been, if the yeast had not been subjected to the light (see Fig. 2). From the values of the metabolism in the light and in the dark, the photoactivity ( $A$ ) of each wave-length may be calculated.

For the *respiratory ferment*,

$$A = \frac{(\Delta p/\Delta t)_l - (\Delta p/\Delta t)_d}{(\Delta p/\Delta t)_d} \times 100$$

where  $A$  = photoactivity;  $(\Delta p/\Delta t)_l$  = change in pressure due to respiration in the light for the period  $\Delta t$ ; and  $(\Delta p/\Delta t)_d$  = change in pressure due to the respiration in the dark for  $\Delta t$ .

And for the *Pasteur enzyme*,

$$A = \frac{(\Delta p/\Delta t)_d - (\Delta p/\Delta t)_l}{(\Delta p/\Delta t)_d} \times 100$$

where  $(\Delta p/\Delta t)_d$  = change in pressure due to fermentation in the dark for  $\Delta t$ ; and  $(\Delta p/\Delta t)_l$  = change in pressure due to fermentation in the light for  $\Delta t$ .

The ratio of the absorption at the wave-length used to the absorption at  $436\text{ m}\mu$  is determined individually for each wave-length. During each experiment before and after illumination at the wave-length under investigation, the photoactivity of two different intensities at  $436\text{ m}\mu$  is determined. From these values a calibration curve is constructed (see Fig. 2). With the aid of

this curve it is possible to find the intensity at 436  $m\mu$  which would produce the same photoactivity as does the wave-length under investigation.

At least two and as many as eleven experiments were conducted at each of the twenty-three wave-lengths for each of the two enzymes. The mean of the results obtained for each wave-length

TABLE II

*Photochemical Effect at 436  $m\mu$  versus That at 589  $m\mu$  on CO Inhibition of Respiration and of Pasteur Reaction in Bakers' Yeast*

Time	Light		Manometer deflections	
	Wave-length	Intensity	Experiment I, respiration (-)	Experiment II, fermentation (+)
<i>min.</i>	<i>mμ</i>	<i>gm. calories per sq.cm. per min.</i>	<i>mm. O<sub>2</sub></i>	<i>mm. CO<sub>2</sub></i>
0				
5			4.17	10.80
10	436	$0.5 \times 10^{-4}$		
15	436		5.68	8.84
20	436		5.60	8.89
25				
30			4.00	11.58
35	589	$2.3 \times 10^{-4}$		
40	589		6.30	7.21
45	589		6.20	7.25
50				
55			3.67	11.80
60	436	$1.4 \times 10^{-4}$		
65	436		6.05	7.24
70	436		5.95	7.28
75				
80			3.43	12.35

has been plotted on the final curves. The individual scattering at one wave-length was similar to that at 560  $m\mu$  described below in the discussion. The data for two experiments at 589  $m\mu$  are presented in Table II.

From Fig. 2, in the bottom half of which are plotted the respiratory  $O_2$  and the fermentative  $CO_2$  against time, there are obtained the values for  $(\Delta p/\Delta t)_d$  at the time when  $(\Delta p/\Delta t)_i$  was being measured. With these values and the experimentally determined  $(\Delta p/\Delta t)_i$  values, the photoactivity ( $A$ ) may be calculated (Table III).

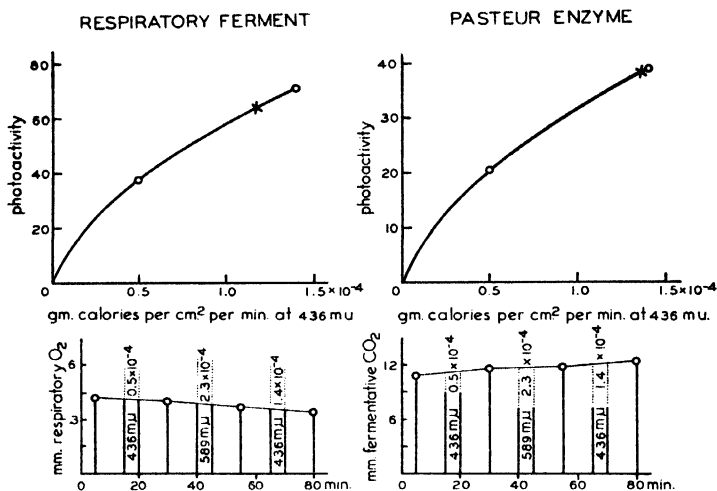


FIG. 2. Determination of  $\beta_{589}/\beta_{436}$  for the respiratory ferment and for the Pasteur enzyme. The graphs in the lower part of the figure represent the changes in metabolism with time. The dotted lines indicate the effect of the blue Hg line and of the yellow Na line on increasing the respiration and on decreasing the fermentation. The line running horizontally and connecting the circles indicates what the metabolic rate would have been if the cells had been kept in the dark. The photoactivity curves in the upper part of the figure indicate at  $\times$  the intensity at 436  $m\mu$  which would have produced the same photoactivity as did  $2.3 \times 10^{-4}$  gm. calorie at 589  $m\mu$ .

TABLE III  
Photoactivity at 436  $m\mu$  versus That at 589  $m\mu$   
Calculated from data in Table II and Fig. 2.

	Time	Light		$(\Delta p/5 \text{ min.})_d$	$(\Delta p/5 \text{ min.})_l$	A
		Wave-length	Intensity			
	min.	$m\mu$	gm. calorie per sq. cm. per min.	mm.	mm.	per cent
Respiratory ferment	15	436	$0.5 \times 10^{-4}$	4.10	5.68	38.5
	20	436	$0.5 \times 10^{-4}$	4.08	5.60	37.3
	40	589	$2.3 \times 10^{-4}$	3.85	6.30	63.6
	45	589	$2.3 \times 10^{-4}$	3.78	6.20	64.0
	65	436	$1.4 \times 10^{-4}$	3.53	6.05	71.4
	70	436	$1.4 \times 10^{-4}$	3.50	5.95	70.0
Pasteur enzyme	15	436	$0.5 \times 10^{-4}$	11.02	8.84	19.8
	20	436	$0.5 \times 10^{-4}$	11.25	8.89	21.0
	40	589	$2.3 \times 10^{-4}$	11.68	7.21	38.3
	45	589	$2.3 \times 10^{-4}$	11.71	7.25	38.1
	65	436	$1.4 \times 10^{-4}$	11.99	7.24	39.6
	70	436	$1.4 \times 10^{-4}$	12.11	7.28	38.4

In Fig. 2 the photoactivity at 436  $m\mu$  is plotted against the intensity at 436  $m\mu$ . From these curves it is found that an intensity of 2.3 gm. calories per sq.cm. per min. at 589  $m\mu$  exerts a photoactivity equivalent to 1.17 gm. calories at 436  $m\mu$  in the instance of the respiratory ferment and to 1.34 gm. calories for the Pasteur enzyme. The relative absorption coefficient at 589  $m\mu$  may then be calculated.

TABLE IV  
*Relative Absorption Coefficients of Pasteur Enzyme and of Respiratory Ferment in Bakers' Yeast*

Wave-length	Light source	Pasteur enzyme	Respiratory ferment	Wave-length	Light source	Pasteur enzyme	Respiratory ferment
$m\mu$		$\beta_{\lambda}/\beta_{436}$	$\beta_{\lambda}/\beta_{436}$	$m\mu$		$\beta_{\lambda}/\beta_{436}$	$\beta_{\lambda}/\beta_{436}$
407	Sr	0.18	0.18	522	Cu	0.03	0.03
425-470	Cu	0.74	0.78	525	Sr	0.02	0.02
430	Cu	2.5*	2.5*	546	Hg	0.06	0.06
436	Hg	1.00	1.00	553	Mg	0.09	0.17
457	Mg	0.37	0.35	560	Ca	0.08	0.21
460	Li	0.32	0.30	578	Hg	0.17	0.16
487	Sr	0.01	0.01	582	Sr	0.26	0.21
494	Mg	0.04	0.02	589	Na	0.44	0.39
497	Sr	0.06	0.03	597	Sr	0.23	0.21
497	Li	0.06	0.03	610	Li	0.03	0.03
515	Cu	0.11	0.15	640-650	Ca	0.00	0.00
517	Mg	0.06	0.07	640-655	Sr	0.00	0.00

\* Calculated; for the method, see Stern and Melnick (3).

For the respiratory ferment,

$$\frac{\beta_{589}}{\beta_{436}} = \frac{1.17 \times 10^{-4} \times 436}{2.3 \times 10^{-4} \times 589} = 0.38$$

And for the Pasteur enzyme,

$$\frac{\beta_{589}}{\beta_{436}} = \frac{1.34 \times 10^{-4} \times 436}{2.3 \times 10^{-4} \times 589} = 0.43$$

### Results

With the technique outlined above the relative photochemical absorption coefficients at twenty-three wave-lengths in the visible region of the spectrum have been determined (Table IV). From

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these values the relative absorption spectra of the CO compounds of the Pasteur enzyme and of the respiratory ferment in the yeast cell have been plotted (Figs. 3 and 4).

No source of intense monochromatic light was available at the very peak of the main absorption band (430 m $\mu$ ). However, from the contour of the rest of the curve and from the  $\beta$  values obtained

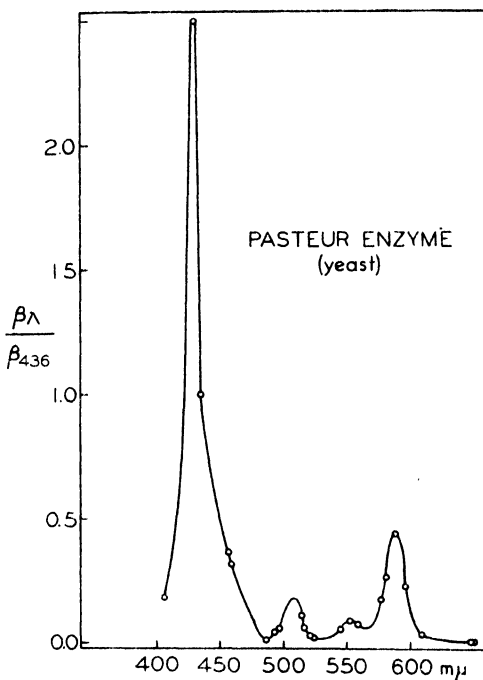


FIG. 3. Relative photochemical absorption spectrum of the Pasteur enzyme CO compound in bakers' yeast.

when the entire blue portion of the spectrum of the Cu-carbon was used, the approximate position and height of the  $\gamma$ -band were determined and the maximum located at 430 m $\mu$ . The procedure followed that previously reported in the construction of the spectrum of the Pasteur enzyme in retina (3).

### DISCUSSION

Previous work in Warburg's (1, 2) and in this laboratory (3) has demonstrated that the patterns of the spectra of the respira-

tory ferment and of the Pasteur enzyme are characteristic for iron-porphyrin proteins. The close chemical relationship between the two enzymes is seen from the present experiments in which both spectra have been charted for the same cell (bakers' yeast): The steep Soret bands or  $\gamma$ -bands of the enzymes coincide in their position at  $430\text{ m}\mu$ , and the low  $\beta$ -bands at  $510\text{ m}\mu$  are

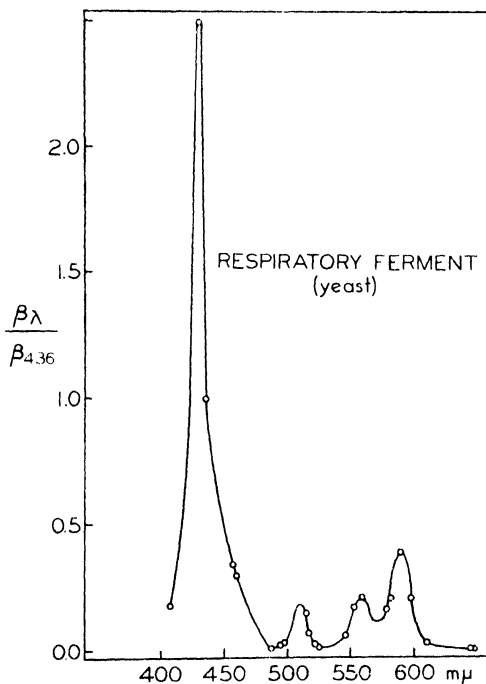


FIG. 4. Relative photochemical absorption spectrum of the respiratory ferment CO compound in bakers' yeast.

also very similar. However, there is a distinct difference in the structure of the  $\alpha$ -bands, the main absorption of which is at  $589\text{ m}\mu$ . Whereas only a slight kink is present in the spectrum of the Pasteur enzyme at  $553\text{ m}\mu$ , a secondary absorption band at  $560\text{ m}\mu$  in the spectrum of the respiratory ferment indicates the non-identity of the two enzymes (Fig. 5).

*Difference between Spectra of the Two Enzymes at  $560\text{ m}\mu$* —In view of the close similarity of the two photochemical spectra here

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reported and the fact that the chief difference consists in the presence of a low, additional band with a maximum at 560  $m\mu$  in the instance of the respiratory ferment, the case for or against the identity of the two catalysts rests on the reality of this band at 560  $m\mu$ . For this reason a fuller discussion of this point is deemed desirable.

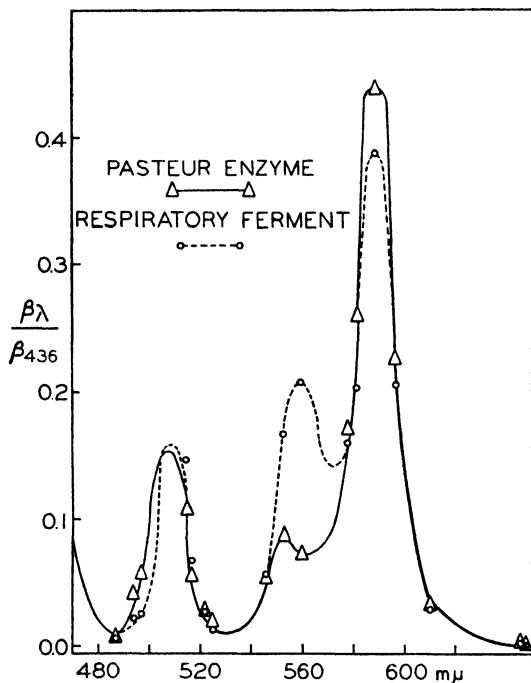


FIG. 5. Photochemical absorption spectra of the CO compounds of the Pasteur enzyme and of the respiratory ferment in bakers' yeast in the region from 470 to 650  $m\mu$ .

The data used for the calculations of the relative absorption coefficients at 560  $m\mu$ , which were derived from six different observations for the respiratory ferment and from nine in the case of the Pasteur enzyme, were subjected to statistical treatment as outlined by Fisher (8).<sup>2</sup> For the respiratory ferment  $\beta_{560}/\beta_{436}$  was

<sup>2</sup> The statistical treatment was kindly performed by Dr. Alfred E. Wilhelmi.

experimentally found to be 0.25, 0.22, 0.21, 0.20, 0.185, 0.175, with a mean value of  $0.207 \pm 0.011$ , or  $0.21 \pm 0.01$ . For the Pasteur enzyme the experimental  $\beta_{560}/\beta_{436}$  values were 0.10, 0.09, 0.085, 0.08, 0.07, 0.07, 0.07, 0.065, 0.055, with a mean value of  $0.076 \pm 0.006$ , or  $0.08 \pm 0.01$ . The difference is  $0.130 \pm 0.014$ , and since it is more than 9 times its standard error, the possibility that the two series of observations are drawn from a single population is almost negligible. Therefore the data warrant the conclusion that the difference here reported in the absorption at  $560\text{ m}\mu$  is significant. Moreover it is also evident from Table IV and Fig. 5 that at  $553\text{ m}\mu$ , which forms part of the band having its maximum at  $560\text{ m}\mu$ , the respiratory ferment absorbs twice as much light as the Pasteur enzyme.

Additional evidence was obtained which also indicates a difference between the two spectra at  $560\text{ m}\mu$ . If a wave-length with a relative absorption coefficient smaller than that at  $436\text{ m}\mu$ , which is arbitrarily set at 1.00, is fixed as the reference wave-length, the absorption at  $560\text{ m}\mu$  should still be about 2.7 times greater for the respiratory ferment than for the Pasteur enzyme. This has been verified experimentally.

When the photoactivity of the radiation of the Ca-carbon from 428 to  $459\text{ m}\mu$  (maximum intensity at  $448\text{ m}\mu$ ) is directly compared with that at  $560\text{ m}\mu$  (isolated from the same Ca-carbon spectrum), it is found that the ratio  $\beta_{560}/\beta_{448}$  for the respiratory ferment is 3 times that for the Pasteur enzyme. For the Pasteur enzyme  $1.56 \times 10^{-4}$  gm. calorie at 428 to  $459\text{ m}\mu$  has the same photoactivity as  $11.40 \times 10^{-4}$  gm. calorie at  $560\text{ m}\mu$ . In the case of the respiratory ferment  $1.52 \times 10^{-4}$  gm. calorie at 428 to  $459\text{ m}\mu$  is equivalent to  $3.63 \times 10^{-4}$  gm. calorie at  $560\text{ m}\mu$ . From these values  $\beta_{560}/\beta_{448}$  is found to be 0.11 for the Pasteur enzyme and 0.335 for the respiratory ferment. As the relative absorption coefficient at  $448\text{ m}\mu$  is the same for both enzymes, the data indicate that at  $560\text{ m}\mu$  the absorption of the respiratory ferment is 3-fold that of the Pasteur enzyme.

*Photochemical Spectra of Pasteur Enzyme and of Cytochrome Oxidase in Rat Tissue*—As the photochemical spectrum of cytochrome oxidase, which is generally identified with the respiratory ferment, has recently been charted in rat heart muscle (9), it is now possible to compare its spectrum with that of the Pasteur



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enzyme measured in the retina of that animal (3). As in yeast, the spectra are similar to each other with differences being located in the long wave region. Both enzymes possess steep  $\gamma$ -bands at  $450\text{ m}\mu$  and small  $\beta$ -bands at  $510\text{ m}\mu$ ; but the  $\alpha$ -band in the yellow of the Pasteur enzyme is situated at  $578\text{ m}\mu$ , whereas the corresponding maximum of cytochrome oxidase is located at  $589\text{ m}\mu$ . Moreover the spectrum of cytochrome oxidase has a definite hump at  $560\text{ m}\mu$  which is lacking in that of the Pasteur enzyme. In the spectrum of the respiratory ferment in bakers' yeast this increase in specific absorption is accentuated so that a small band is actually measurable at  $560\text{ m}\mu$ . In the instance of the Pasteur enzyme, in both rat retina and in yeast, there is very little absorption in this region. The spectra obtained for the Pasteur enzymes in retina and in yeast on the other hand are sufficiently different to justify the conclusion that they too must differ either with regard to their hemin group or their protein component.

*Reactions to Chemical Agents*—The similarity in the spectra of the respiratory ferment and Pasteur enzyme in yeast helps to explain why it has been widely assumed that respiration checks fermentation. The spectra show that the two enzymes are very similar chemically; therefore they would be expected to react in a similar fashion with compounds having an affinity for the hemin group. Thus the inhibition of respiration and the concomitant increase in aerobic fermentation in the presence of cyanide or CO are readily explained by the inhibition of *both* the respiratory ferment and the Pasteur enzyme.

In some tissues, however, it is possible to distinguish between the two catalysts by inhibiting only one of them with specific reagents. For example, ethyl isocyanide (10) and CO (4) in certain instances have been shown to inhibit selectively the Pasteur reaction. Low  $\text{O}_2$  tensions may selectively inhibit either the Pasteur reaction (11) or respiration (12). These observations may be explained by the different affinities which the respiratory ferment and the Pasteur enzyme<sup>3</sup> have for the inhibiting agents and for  $\text{O}_2$  (3).

<sup>3</sup> Although the compound reacting with ethyl isocyanide in Warburg's experiments (11) has not yet been identified with the Pasteur enzyme as defined by its CO spectrum, it appears likely that the same heavy metal catalyst is involved in both reactions. This is supported by the fact that the iron-porphyrin protein hemoglobin, which shares basic chemical features with the Pasteur enzyme, reacts both with CO and  $\text{C}_2\text{H}_5\text{NC}$  (13).

## SUMMARY

The absorption spectra of the CO derivatives of the respiratory ferment and of the Pasteur enzyme have been charted in bakers' yeast by Warburg's photochemical method. Both enzymes exhibit a spectrum characteristic of pheohemin proteins. The steep  $\gamma$ -bands in the blue at 430  $m\mu$  coincide and the small  $\beta$ -bands in the blue-green at 510  $m\mu$  are also similarly located. However, the structure of the spectra in the green and yellow is significantly different: whereas both enzymes have a strong  $\alpha$ -band at 589  $m\mu$ , the spectrum of the respiratory ferment shows an additional, secondary maximum at 560  $m\mu$ .

There thus appear to exist within the same cell two hemin-containing enzymes capable of reacting with molecular oxygen. One, the *respiratory ferment*, is instrumental in the oxidation of metabolites; the other, the *Pasteur enzyme*, controls the inhibition of fermentation by oxygen.

The author is indebted to Dr. Kurt G. Stern for his stimulating interest in this work.

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# THE LIPIDS OF DIATOMS\*

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In the course of an investigation of the chemical components of primitive, autotrophic organisms ((1) and unpublished work), the fatty constituents of marine diatoms<sup>1</sup> have been examined. Beyond the analytical characterization of the oil of *Nitzschia closterium* (2) and a quantitative study of its individual fatty acids (3), chemical information pertaining to the lipids of diatoms is relatively scanty. In the study here reported, the surprising finding was made that the lipids extracted from a sample of mixed diatoms contained the major part of their fatty acids in the free form. As this sample had been dried before analysis, it was thought that this might be ascribable to postmortem lipolysis. However, diatoms placed under ether or acetone immediately after collection showed the same peculiarity, which appears to be unique.

The samples first examined were collected off Woods Hole by Dr. G. L. Clarke (4) of the Biological Laboratories of Harvard University. No pronounced differences were observed in the chemical composition of the lipid from diatoms collected at different seasons and with counts (made by Miss Lois Lillick) which indicated widely differing biological composition. Through the kindness of Dr. A. C. Redfield it was later possible to examine a quantity of the marine diatom *Nitzschia closterium* grown in pure culture (5), and here again the bulk of the fatty acids was likewise found to exist in the free state. The results of the analyses are recorded in Table I.

\* This investigation was made possible by a grant from the Carnegie Corporation of New York.

<sup>1</sup> The collection by Dr. George L. Clarke of marine diatoms for this work was made possible through the kind cooperation of Dr. Henry B. Bigelow, Director of the Woods Hole Oceanographic Institution.

In contrast to the behavior of most animal and vegetable oils, that of diatoms showed a decreased amount of free fatty acid and an increased amount of neutral fat when the sludge of organisms, as collected, was allowed to stand undisturbed for some days. One portion of a catch collected April 14 to 15, 1938, was analyzed after 8 days. The lipid contained 67 per cent of free fatty acid. Other portions of the same original suspension were allowed to stand at room temperature for 6 months alone and in the presence of sea bottom mud under aerobic and anaerobic conditions. Analyses were carried out upon uniform samples of the suspen-

TABLE I  
*Composition of Diatom Lipids*

	Per cent of total lipid			
	Sample A	Sample B	Sample C	Sample D
Free acids.....	82	70	59	80
Combined acids.....	5	1	17	17
Unsaponifiable.....	12	29		
Alcohols.....			7	3
Hydrocarbons.....			14	3

Sample A, diatoms (85 per cent *Rhizosolenia alata*) dried after collection (September, 1937); they contained 8 per cent lipid, 46 per cent ash (dry basis). Sample B, mixed marine diatoms, covered with ether on collection (September, 1937); 10 per cent lipid, 50 per cent ash. Sample C, mixed marine diatoms, in water as collected (April 27, 1938); analyzed after 5 days: 15 per cent lipid, 36 per cent ash. Sample D, *Nitzschia closterium*, grown in sterile culture, immediately covered with acetone; 5 per cent lipid, 42 per cent ash.

sions (Table II). The material which had been allowed to stand alone, without exclusion of air, showed a 50 per cent increase in total lipid (from 64 to 95 mg. per 100 gm. of suspension). This increase occurred mainly in the unsaponifiable fractions, and there was an almost complete disappearance of the free fatty acids. Similar, though less pronounced, changes occurred in the presence of sea bottom mud.

As this finding was of interest in its possible bearing on the hypothesis (6, 7) that petroleum originates in deposits of marine plankton, a second experiment (Table III) was carried out a year later, with diatoms collected early in April, 1939. In this case the

control analysis was performed on a portion of the suspension which had immediately been treated with an equal volume of acetone. However, after 6 months the total lipid content had fallen to less than one-half of its original level; while the total hydrocarbon content of the diatoms remained essentially unchanged, it constituted an increased fraction of the total lipid. These differences may possibly be due to differences in the putre-

TABLE II  
*Lipid Fractions Isolated from Diatom Suspensions and Mud*

	100 gm. diatom suspension		100 gm. mud		100 gm. diatom suspension + 100 gm. mud	
	8 days in air	6 mos. in air	8 days in air	6 mos. in air	6 mos.	
	mg.	mg.	mg.	mg.	In air	In nitrogen
Free acids.....	43	1	8	2	10	5
Combined acids.....	13	34	6	11	6	7
Alcohols.....	2	14	2	4	7	4
Hydrocarbons.....	3	23	12	6	32	19

TABLE III  
*Lipid Fractions per 100 Gm. of Dry Diatoms*

	Diatoms, fresh	Diatoms, 6 mos. aerobic
	gm.	gm.
Free acids.....	4.6	1.1
Combined acids.....	1.0	0.5
Alcohols.....	0.3	0.2
Hydrocarbons.....	1.4	1.3

factive organisms; in this second experiment a mold<sup>2</sup> grew in large amounts.

The hydrocarbon fraction obtained from a relatively large catch of diatoms (Sample C, Table I) yielded a crystalline paraffin hydrocarbon, possibly impure hentriacontane, the presence of which in the lipids of some higher plants has been reported by various investigators. The unsaponifiable material also contained a

<sup>2</sup> Kindly identified as *Mucoraceae cunninghamella* by Dr. E. D. Delamater of this School.

digitonin-precipitable fraction from which there was obtained a crystalline product that appeared to consist of sitosterols.

It was thought that the presence of so much free fatty acid in the living diatoms, in contrast to other organisms, might be associated with the occurrence of a high proportion of free silicic acid. Analyses were accordingly made of the lipids and residual tissues of samples of mixed marine diatoms, a siliceous fresh-water sponge,<sup>3</sup> and two marine sponges,<sup>4</sup> all of which had been covered with acetone immediately after collection. The results (Table IV) indicated no such parallelism; the sponge oils all contained appreciable amounts of free fatty acid, but the level was not higher in the siliceous than in the calcareous sponges.

TABLE IV  
*Fatty Acid, Silica, and Calcium in Diatoms and Sponges*

Organism	Total lipid	Free fatty acids	Total silica	Total calcium
	per cent, dry basis	per cent total acids	mm per gm. dry, extracted tissue	mm per gm. dry, extracted tissue
Diatoms (mixed marine).....	15	78	6.4	1.7
Siliceous sponge, <i>Spongilla lacustris</i> .....	9	31	6.9	0.4
Red sponge, <i>Tedania ignis</i> .....	4	41	1.0	6.3
Cap " <i>Hircinia campana</i> ....	5	17	0.1	2.4

#### EXPERIMENTAL

The methods employed are exemplified by the treatment to which the diatom Sample C (Table I) was subjected.

**Total Lipid**—The suspension was filtered; the filtrate was shaken with ether and the residue on the filter exhaustively extracted with ether. The combined ethereal solutions were evaporated to dryness; the residue was taken up in petroleum ether and filtered. This filtrate contained 17.6 gm. of lipid; the weight of dry, ether-insoluble residue was 96 gm.

**Free Fatty Acids**—The above petroleum ether solution (about 1

<sup>3</sup> Collected by Dr. P. Conger at the Lake Laboratory of the University of Wisconsin.

<sup>4</sup> Collected by Dr. Hugh H. Darby at the Tortugas Laboratory of the Carnegie Institution of Washington.

liter) was shaken repeatedly with small portions of a 0.5 per cent solution of potassium carbonate in 50 per cent methyl alcohol, until no further acids were extracted. The upper layer contained 6.88 gm. of neutral lipid; the combined aqueous-alcoholic solutions, on acidification and extraction with petroleum ether, yielded 9.88 gm. of free fatty acids. These were separated by means of the lead salts into 1.755 gm. of saturated and 7.089 gm. of unsaturated acids.

*Fractionation of Fatty Acids*—The mixture of saturated fatty acids (1.755 gm.) from the free acid fraction showed on titration an average equivalent weight of 253. The material was converted into the methyl esters, which were distilled under 0.005 mm. pressure. A series of six fractions was collected, the saponification equivalents of which, according to the method of Lovern (8), showed the presence of 60 per cent palmitic acid, 36 per cent stearic acid, and only minute proportions of lower and higher homologues.

The unsaturated acids from the same fraction had an average equivalent weight of 296. They were hydrogenated in petroleum ether solution with a palladium catalyst; the uptake corresponded to 1.96 moles of hydrogen per mole of acid. The resulting mixture of saturated acids was separated into a solid fraction (2.598 gm.) and a liquid fraction (4.304 gm.) of which the lead salts were respectively insoluble and soluble in methyl alcohol. These were converted into the methyl esters and fractionated by Lovern's method.

	C <sub>14</sub>	C <sub>16</sub>	C <sub>18</sub>	C <sub>20</sub>	C <sub>22-24</sub>	C <sub>24-30</sub>
Solid acids.....	7	29	7	20	37	
Liquid acids.....		26	13	19	31	11

These results resemble in general those secured by Lovern (3) with the total acids of *Nitzschia closterium*.

*Combined Fatty Acids*—To the neutral lipid in 200 cc. of petroleum ether, 200 cc. of 4 per cent methyl alcoholic potassium hydroxide were added, and the mixture was shaken intermittently for 2 days, whereupon 200 cc. of water were added. The lower layer was separated, washed with petroleum ether, acidified, and extracted with petroleum ether. The resulting fatty acids weighed 2.806 gm. These were separated into 0.478 gm. of saturated and



2.109 gm. of unsaturated acids. The unsaponifiable material weighed 3.658 gm.

The acid aqueous solution, after the removal of fatty acids, yielded 0.315 gm. of glycerol, identified as the tribenzoate (m.p. and mixed m.p. 72°).

*Alcohol Fraction*—To the carefully dried unsaponifiable material, dissolved in 20 cc. of dry pyridine, 10 gm. of succinic anhydride were added. The mixture was warmed on the steam bath for 1 hour, allowed to stand overnight at room temperature, and poured into 400 cc. of water. After an hour the resulting suspension was extracted with ether and the ethereal solution repeatedly washed, first with 0.5 N sulfuric acid and then with dilute sodium carbonate. The alkaline washings were acidified. The 1.448 gm. of crude acid succinic esters extracted by ether were subjected to alkaline hydrolysis, when 1.189 gm. of alcohols were secured.

*Hydrocarbon Fraction*—The neutral ethereal solution from which the acid succinates had been removed contained 2.405 gm. of hydrocarbon fraction (which comprised any non-hydroxylated ketones that may have been present). This was dissolved in hot methyl alcohol, and the solution was chilled with solid carbon dioxide and centrifuged at 0°. Further crops of crystalline material were secured from the mother liquor. The solid material, recrystallized from ethyl acetate, yielded a pale yellow, crystalline product, m.p. 57°, which was distilled at 150° and 0.02 mm. pressure. The colorless distillate was recrystallized to a constant melting point of 59°. The product (0.269 gm.) was inert towards bromine and hot concentrated sulfuric acid. Its composition and molecular weight correspond with those of hentriacontane, but the melting point is 9° lower.

$C_{31}H_{64}$ .	Calculated.	C 85.22,	H 14.78,	mol. wt. 436
	Found.	" 84.9,	" 14.9,	" " 431

*Sterol Fraction*—The insoluble digitonide from the unsaponifiable fraction (1.0 gm.) of Sample A of Table I weighed 1.0 gm.; it was decomposed according to the method of Schoenheimer and Dam (9) and yielded 103 mg. of a sterol which crystallized in long needles from petroleum ether. This product was repeatedly recrystallized from 95 per cent ethyl alcohol, until no further change in melting point (138°) was observed. It showed  $[\alpha]_D =$

−41.6° (in chloroform), and gave a blue-green color in the Liebermann-Burchard reaction but none in the Rosenheim test. The acetate had a melting point of 132°,  $[\alpha]_D = -22.9^\circ$  ( $\text{CHCl}_3$ ).

$\text{C}_{31}\text{H}_{52}\text{O}_2$ .	Calculated.	C 81.51,	H 11.48
	Found.	" 81.3,	" 10.7

#### SUMMARY

1. The lipids of freshly collected marine diatoms contain a very high per cent of free fatty acid, apparently irrespective of species.

2. When a suspension of diatoms is allowed to stand for 6 months, the content of free acids falls markedly, and the content of hydrocarbon may rise.

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# THE PASSAGE OF PROTEIN MOLECULES THROUGH THE GLOMERULAR MEMBRANES\*

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We conceive of the glomerular membrane as a thin composite structure, the complex molecules of which are so arranged as to form liquid-filled passages or pores through which a filtrate can be expressed from the blood plasma. Analyses of the glomerular filtrate obtained from Amphibia and study of the renal excretion of the polysaccharide inulin (1) and of different proteins have yielded information with which the permeability of the membrane may be defined and thus by inference a description of its character approached. Normally, serum proteins do not pass through it. The smallest of these is serum albumin (molecular weight 70,000); hence we say tentatively that all of the pores of the membrane are smaller than the diameter of the serum albumin molecule. Aiming to decide whether other factors than molecular weight and size determine the passage or non-passage of a substance through the glomerulus, we have studied the glomerular excretion of several proteins, the molecular weights of which lie between those of inulin and serum albumin.

Mainly two types of experiments have been performed: (a) "direct" experiments in which glomerular fluid collected from Bowman's capsule was analyzed for protein by an ultramicro-method which will be described. The collections were made from amphibian kidneys (chiefly of *Necturi*) during perfusion with protein solutions; (b) "indirect" experiments (chiefly on frogs) in

\* The expenses of this work have been defrayed in large part from a grant by the Commonwealth Fund of New York. A preliminary report was made to the Physiological Society of Philadelphia in October, 1940 (see *Am. J. Med. Sc.*, **200**, 847 (1940)).

which urine was collected from the ureters during perfusion of a solution containing protein and inulin and analyzed for these two substances.

In addition we have performed a few experiments in which the rate and completeness of excretion of protein injected into normal dogs was compared with that of inulin similarly injected.

*Procedure for Perfusion Experiments*—Proteins, sometimes together with inulin, were made up in frog Ringer's solution (2) containing no glucose or glycine. The oxygenation was accomplished in most cases by bubbling a mixture of 98.5 per cent oxygen and 1.5 per cent carbon dioxide through the solution by means of a sintered glass bubbling tube. In several experiments solutions were subjected to a pressure of 10 atmospheres of the gas mixture mentioned above for approximately 45 minutes for oxygenation. The pH of the perfusion fluid was approximately 7.4 except when otherwise noted. After oxygenation the fluids were filtered through rapid filters into perfusion bottles.

The various proteins used were crystalline albumin of hen's and duck's eggs, Palmer's lactoglobulin (3), amorphous and crystalline (zinc) insulin, all of which have molecular weights of 34,000 to 46,000 (4-7)<sup>1</sup> and are placed in the 35,200 group of Svedberg's classification, purified protein derivative (PPD-49609) from tuberculin (8, 9) with a weight of about 14,500, and, as a control, crystalline horse serum albumin. Attempts were also made to use the protamine, salmine (the molecular weight may be 5600 (10)), but this material proved toxic to the kidney. Details concerning the preparation of proteins<sup>2</sup> and protein solutions are as follows:

*Hen's egg albumin* was crystallized at least three times by the Sørensen (ammonium sulfate) method or by the Kekwick and Cannan (11) (sodium sulfate) method. After removal of sulfate by dialysis, some of the solution was used immediately for per-

<sup>1</sup> Since it is impossible to quote the large number of original papers, we are referring to recent publications in which data from such sources are assembled and discussed.

<sup>2</sup> It is a pleasure to acknowledge our gratitude to Dr. Florence Seibert of the Henry Phipps Institute for the purified protein derivative of tuberculin, to Eli Lilly and Company for insulin, to Sharp and Dohme for salmine, and to Professor B. M. Hendrix for sending one lot of partially purified duck's egg albumin from Texas at a time when we were unable to obtain duck's eggs.

fusion; the larger part of it, however, was lyophilized in small containers and kept *in vacuo* until used. When lyophilized albumin was used, it was first dissolved in water and any protein which did not dissolve at this pH (the isoelectric point) was filtered or centrifuged off. The pH of the solution was raised to at least 6 by the addition of sodium hydroxide before the various constituents of the Ringer's solution were added. The pH of the oxygenated solutions was 7.4. One portion of lyophilized egg albumin, made up into perfusion fluid and aerated as usual, was used for sedimentation in the ultracentrifuge. We are indebted to Dr. Bauer and Dr. Pickels of the International Health Division of the Rockefeller Foundation for this determination which showed the protein to be homogeneous and to have a sedimentation constant  $S_{20^\circ} \times 10^{-13} = 3.51$  which compares well with that of 3.55, the latest figure from the Svedberg laboratory (7). Photographs of the boundary movement are shown in Fig. 1.

The same methods of purification were applied to duck's egg albumin but the crystals obtained, although pure white, were never clear cut as in the case of hen's egg albumin.

*Lactoglobulin* was prepared as described by Palmer (3) with the use of ammonium sulfate and recrystallized twice. The crystals were beautifully clear cut tabular crystals and were kept under water, preserved with toluene. They were washed with water before being dissolved in the chlorides of Ringer's solution.

*Insulin*.—Both amorphous and crystalline zinc insulin were supplied by Eli Lilly and Company. Since Sjögren and Svedberg (see (7)) had shown that pH 7 is the upper limit of the stability range for insulin, all perfusion fluids in insulin experiments were adjusted to this value instead of pH 7.4.

*PPD-49609*.—This protein (8) supplied by Dr. Seibert shows a dry molecular weight of about 14,500 and is highly homogeneous in so far as the ultracentrifuge discloses but heterogeneous by electrophoresis. The material was supplied phosphate-buffered and lyophilized. It was dissolved in water under a partial vacuum to form a clear brown solution. In some experiments the protein was simply made up with the usual chlorides and bicarbonate of perfusion fluid, the phosphate being omitted. In other cases the protein-phosphate-chloride solution was run through a Jena bacterial filter before use, since a fine precipitate was removed in

this way. This precipitate may have formed by interaction with the various chlorides. It was not visible in suspension but formed as a thin brown layer on the filter. A second filtration of the same

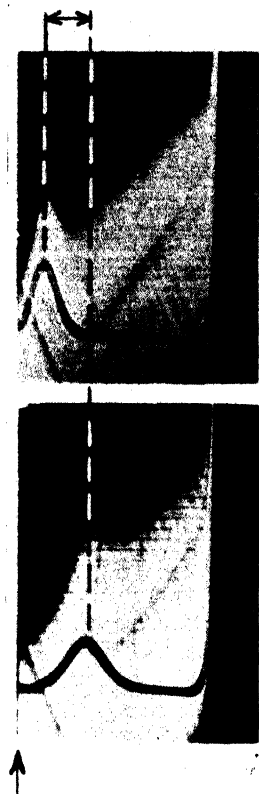


FIG. 1. Sedimentation of egg albumin. The horizontal arrow indicates boundary movement of 2.5 mm. in 1 hour, 23 minutes. The perpendicular arrow indicates the meniscus of the solution. Speed = 720 revolutions per second; centrifugal force = 133,000 gravity.

material showed that almost none of this precipitate was then present.

*Crystalline horse serum albumin* was prepared from fresh horse serum and recrystallized as described by Sørensen. It was lyophilized and formed pale yellow solutions when redissolved.

*Salmine*—"Pure protamine, salmine," Sharp and Dohme, was used.

All the inulin used in these experiments was prepared about 6 years ago by Dr. R. F. Jackson of the Bureau of Standards. The concentrations of protein in perfusion fluids depended to a certain extent on the availability and solubility of the material. It was necessary to make up several hundred cc. of fluid for each experiment. The concentrations ranged from 0.05 to 2.6 per cent. Inulin, when present, was in a concentration of 0.4 to 0.5 per cent.

In the indirect type of experiment the bullfrog kidney was perfused via the aorta with protein-inulin solution at a pressure usually near 24 cm. of water. In a few instances plain Ringer's solution was perfused simultaneously through the renal-portal circulation, but this did not alter the results. Urine was collected from the ureters by cannula and analyzed for protein and inulin. Perfusion fluids were likewise analyzed. Since it has been shown (1) that inulin is completely filtered through the glomeruli of Amphibia, and on the assumption that neither protein nor inulin is secreted or reabsorbed by the tubule cells, the concentration of protein in glomerular fluid was calculated from the results of the above analyses. For example, if the inulin urine to perfusion fluid ratio is 1.2, then the protein must also have been concentrated 1.2 times in passing from capsule to ureter, and the urine protein concentration is therefore divided by 1.2 to obtain the glomerular fluid protein concentration. If, by this process, the concentration of protein in glomerular fluid is found to be only one-half of that in perfusion fluid, it is concluded that the glomerular membrane is only 50 per cent permeable to the protein. For convenience this will be designated as 50 per cent filtration.

In the direct experiments glomerular fluid was collected by the method described by Wearn and Richards (12). Adult *Necturi* were used for this purpose, since the glomeruli are large and samples of the volume necessary for analysis could be collected fairly readily. The animals were placed in 1.5 per cent urethane solution for approximately 15 minutes, brain and spinal cord were then completely pithed, an inflow cannula inserted in the dorsal aorta above the kidney, and the outflow cannula in the postcaval vein. There are certain difficulties inherent in glomerular puncture perfusion experiments in these animals which make the collections



both difficult and treacherous. It is because of these difficulties that some experiments are necessarily discarded and some must be regarded as questionable when conditions are not perfect during the perfusion.

### *Analytical Methods*

*For Experiments on Dogs*—For these experiments urinary protein was determined by the gravimetric method of Folin and Denis (13); inulin as previously described (14).

*For Indirect Experiments on Amphibia*—Inulin was determined as the total reducing substance after the usual protein removal and hydrolysis (see (14)), since neither perfusion fluid nor urine contained glucose. The Somogyi reagent for small quantities of sugar (15) was used for the determination.

Protein was determined by means of the Folin and Ciocalteu (16) phenol reagent as suggested by Greenberg (17). The Evelyn photoelectric colorimeter was used for these microdeterminations, modified to fit our needs. Standardization curves were plotted with protein solutions, the protein content of which had originally been determined by Kjeldahl nitrogen analysis<sup>3</sup> (Wong's (18) persulfate method). For the standardization, 0.05 cc. samples of protein solutions were washed into 6 cc. volumes of water in a series of tubes. To each of these and also to a water blank was added, with swirling, 0.25 cc. of 5 N NaOH. 10 minutes after the addition of alkali to the first tube, the addition of phenol reagent was begun. 0.4 cc. was added to each tube rapidly but drop by drop and with continuous swirling. After 15 minutes had been allowed for color development, readings were taken, with the 6 cc. aperture of the colorimeter and the No. 620 filter. 0.05 cc. of 1.8 per cent egg albumin produced full scale deflections.

Perfusion fluids and urines were made up for color development as described for the standard solutions, and readings were taken, and evaluated as protein concentrations on the curve. The error of these determinations was approximately 3 per cent. When it was necessary to take samples larger or smaller than 0.05 cc., corrections were made in the volume of water used, so that the final volumes remained the same. Proteins which contain more

<sup>3</sup> We wish to acknowledge the assistance of Miss Ethol Shiels for these and other analyses in the standardization of protein.

tyrosine or tryptophane than egg albumin gave relatively higher color development, so that for correct absolute values, the readings were evaluated from curves plotted for these proteins. Ratios of urine concentration to perfusion fluid concentration (and consequently calculated percentage filtration of proteins) were practically identical, however, whether the readings were interpreted from the various curves mentioned or from the curve for egg albumin.

The Weber colorimetric arginine method (19) was used to determine protamine (salmine) in a few experiments. The depth of color was read photoelectrically.

*For Direct Experiments on Amphibia. Ultramicro Protein Determination*—The same method described above was used to determine protein colorimetrically, an ultramicro colorimeter being used for the readings. In this colorimeter, which was built for us by Dr. K. Hartline and Mr. J. Hervey, the potentiometer principle is utilized. The amplified photocell current is "bucked" against a current from a battery source, the null point being indicated by means of a milliammeter. The unique absorption cells, designed for the colorimeter by Dr. Hartline, are illustrated in Fig. 2. This type of cell met the requirements of being quickly and thoroughly cleaned. It is composed of three slides, each 1 mm. in thickness, two of which are shown (*A* and *B*) and the third of which (*A'*) is a mirror image of *A*. Circular grooves are ground into *A* and *A'*, while in *B* there is a circular hole completely through the slide. A small drop of fluid is placed on each of the surfaces inside of the circular grooves, while the three slides are held in one hand, properly oriented so that when they are allowed to come together they will appear as is indicated in *C* of Fig. 2. The volume of fluid held by the cell is approximately 0.025 cc.

The range and accuracy of the colorimeter are shown by the results of readings taken when the cell is simply filled with solutions such as copper sulfate and indigo carmine. With concentrations of copper sulfate from 0.5 to 10 per cent which showed  $-\log$  transmissions from 0.00358 to 0.07196, the readings ranged from 0.0 to 5.0 per cent from the theoretical, averaging 1.7 per cent. In another series of readings on indigo carmine solutions varying in concentration from 0.7 to 50 mg. per cent, the light absorption in this case varying from 4 to 90 per cent, the error

was even lower. This error includes that of the instrument, of cleaning the glass cell, and filling it with solution.

Results on protein solution are not as good as those cited above, since added to these errors are those of the protein method itself (17), of measuring out the various reagents and the sample, as well as the occasional formation of bubbles. Capillary pipettes delivering by centrifugal force were used rather than washout pipettes in order to reduce the chances for bubble formation in

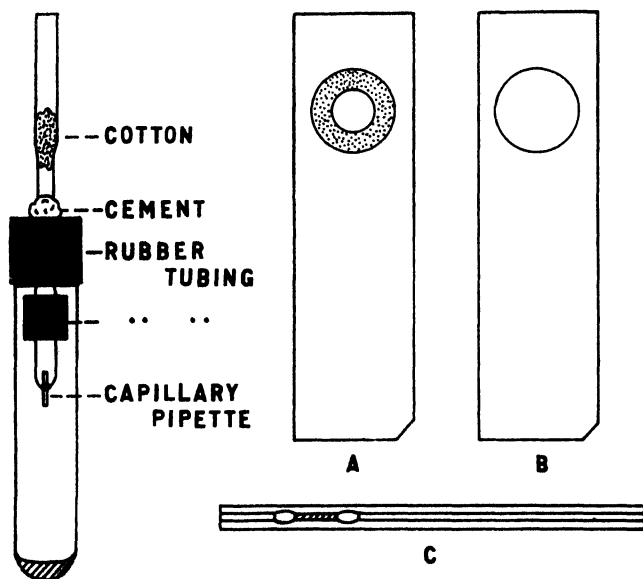


FIG. 2. Left, apparatus for ultramicro protein determination. Right, absorption cell for micro photoelectric colorimeter. A, B, and C are slides, as described in the text. The entire figure is actual size.

these alkaline protein solutions. The manipulation of these pipettes will be described briefly. The protein solution (either standard or experimental) is transferred under the microscope to the capillary pipette holding about 0.5 c.mm. which is mounted in a glass holder similar to that described by Wigglesworth (20) but supplied with shoulders of rubber tubing as shown in Fig. 2. The filled pipette is placed in a sturdy test-tube into which has been measured 0.050 cc. of 0.2 N sodium hydroxide solution. The entire assembly (shown in Fig. 2) is then lowered

into a centrifuge cup and spun rapidly for a few seconds to discharge the sample. The pipette is removed and the fluids mixed by tapping the test-tube gently. After 10 minutes a pipette mounted similarly but made of large capillary tubing (0.6 mm. inside diameter and 11 mm. long) and holding 3 c.mm. is filled with phenol reagent which is delivered as indicated above. 15 minutes later the blue solution is drawn up in a fine tipped pipette and transferred to the absorption cell as previously described. In nineteen single determinations of protein in 0.5 c.mm. samples of 0.2 to 3.8 per cent protein solutions the average error was 4.9 and the range of error 0.0 to 14.7 per cent. In only two of the experiments was the error above 10 per cent.

### *Results*

The results of thirty-two "direct" glomerular puncture experiments and over 50 "indirect" experiments are given in Tables I and II. That there are more figures for hen's egg albumin than for any of the other proteins is due to the fact that, being most easily prepared, it was used as a basis for comparison with each of the others, and for experiments made to test technique.

The direct glomerular puncture experiments show that on the average the concentration of hen's egg albumin in the glomerular filtrate in *Necturi* is 58 per cent of that of the perfusion fluid (range, 38 to 84). The indirect experiments on frogs gave an average value of 57 per cent (39 to 77). This surprisingly close agreement is testimony to the validity of the indirect experiment. Eight different lots of the hen's egg albumin were prepared for these experiments at different times; no significant differences ascribable to different preparations were observed. The concentrations of the protein in the perfusion fluid in different experiments varied from 0.1 to 2.6 per cent. These differences did not affect the percentage filtered. The close agreement of the two methods can be used as evidence that neither inulin nor hen's egg albumin is reabsorbed from the tubules. The conclusion from these experiments is that the permeability of the glomerular membrane to hen's egg albumin is incomplete. It might be justifiable to say that only from 45 to 70 per cent of the surface of the membrane or of the pores in the membrane permits the passage of that protein.

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TABLE I

*Protein Perfusion Experiments on Necturi, Glomerular Punctures (Direct)*

Hen's egg albumin			Duck's egg albumin			Lactoglobulin		
Perfusion fluid	Glomerular fluid	Per cent filtered	Perfusion fluid	Glomerular fluid	Per cent filtered	Perfusion fluid	Glomerular fluid	Per cent filtered
<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
1.16	0.88	76	0.65	0.27	42	1.00	0.70	70
0.94	0.36	38	0.53	0.28	53	0.74	0.57	77
0.77	0.39	51	0.51	0.29	57	0.58	0.51	88
0.68	0.41	60	0.49	0.47	96	0.53	0.54	102
0.66	0.32	49	0.47	0.45	96	0.44	0.37	84
0.66	0.55	83	0.41	0.24	58	0.40	0.41	103
0.56	0.46	82				0.22	0.14	64
0.54	0.29	54				0.17	0.14	82
0.54	0.26	48						
0.48	0.23	48						
0.48	0.24	50						
0.47	0.20	43						
0.45	0.38	84						
0.44	0.22	50						
0.42	0.30	71						
0.42	0.24	57						
0.41	0.19	46						

TABLE II

*Per Cent Filtration of Various Proteins*

Mol. wt.	Protein	By analysis of glomerular fluid; <i>Necturus</i>			By indirect method; frog		
		No. of experiments	Range of results	Average	No. of experiments	Range of results	Average
70,000	Crystalline horse serum albumin	Practically no filtration					
	(Crystalline hen's egg albumin	17	38- 84	58	50	39-77	57
35,200 class	Duck's egg albumin	6	42- 96	67	5	53-58	55
	(Crystalline lactoglobulin	8	64-103	84	8	72-85	79
	Amorphous insulin	1	24	24	1	15-20	18
	(Crystalline zinc insulin				1	22-42	32
14,500	PPD from tuberculin				6	76-95	86

We were interested in trying duck's egg albumin, since there was some evidence that this protein was more homogeneous

electrophoretically than that of the hen's egg (21, 22). Only one component has been demonstrated for the former; the latter, which, it must be said, has been studied more completely, shows more than one. In spite of this both showed approximately the same percentage filtration through the glomeruli. It may be a coincidence that the results of indirect experiments on duck's egg albumin were all within a narrow range (53 to 58 per cent). It happens that two of the direct experiments indicate almost complete filtration. This may mean either that the membranes in these cases were abnormal or that there was an undetected flaw in the technique used during the glomerular puncture. We are of the opinion that, could more experiments be performed, the results of these would be similar to those indicated by the other experiments; namely, 40 to 60 per cent filtered.

The membranes appear to be more permeable to Palmer's lactoglobulin (3), a protein of the same molecular weight group (7) as egg albumin. The filtration percentage is from 72 to 85 in the bullfrog experiments and 64 to 103 in the *Necturus* puncture experiments. Here again we see a larger range in the latter type of experiment, but average values agree fairly well. Although there is an overlapping of the filtration ranges for egg albumin and lactoglobulin, because of the variation from animal to animal, it will be shown later that, whenever the two proteins were perfused through the same kidney, the permeability toward lactoglobulin was always greater.

Insulin is another protein of this molecular weight group. Although the homogeneity as to particle size, especially under the conditions of the experiments, cannot be considered assured, the results may be of interest.<sup>4</sup> In three experiments with amorphous and crystalline zinc insulin (one direct and two indirect) the permeability shown by the membrane toward this protein was only 15 to 24 per cent.

The small molecule, PPD-49609 from tuberculin, showed the highest filtration, as was to be expected from its low molecular weight (14,500) (8). Because of the value of this material, we preferred to use it in several indirect experiments rather than to chance the use of approximately the same amount necessary for a

<sup>4</sup> The same is true of samples of Bence-Jones protein kindly supplied by Dr. Grace Medes of the Lankenau Hospital, Philadelphia, and by Professor D. W. Wilson; these showed filtration values of 20 to 48 per cent.

single puncture experiment. Assuming that the tubules act toward this protein as they do toward the others, we must conclude that the PPD is not quite completely filtered.

Attempts to use salmine were unsuccessful, because the material was too toxic to the kidney even in a concentration of 0.1 per cent. When perfusion began, the membranes seemed to be nearly completely permeable to the protamine, but since permeability to horse serum albumin developed in about 5 minutes and continued even after protamine perfusion had been discontinued, it is dangerous to consider the permeability to protamine as normal. Normally the glomerular membranes were practically impermeable to horse serum albumin, which has a molecular weight almost twice that of egg albumin.

*Additional Information Obtained by Indirect Method*—The indirect method of determining permeability of the glomerular membranes to various proteins is advantageous in several ways: With the same amount of perfusion fluid and in the same time required for the collection of an ultramicro sample (0.5 c.mm.) of glomerular fluid, it is possible to collect several samples of urine large enough (about 0.4 to 1.0 cc.) to permit the analysis for inulin and protein by means of micromethods. Obviously one may perfuse first with one kind of protein, and follow with another, sometimes including as many as four different kinds of proteins in the same experiment while the kidney is still functioning normally. It is possible to observe any alteration in permeability of the membrane due to the passage of protein through it and to observe as well the influence of various drugs on permeability. The results of five experiments of this type are given in Table III. In the first three it is demonstrated that different proteins may be perfused in the same experiment, with proper washout periods between collections, and that approximately the same result is obtained for a certain protein each time it is used. These same experiments show the difference in permeability, in the same kidney, toward the different proteins. There is no evidence here of any influence of a protein on the permeability of the membrane except, possibly, in the case of insulin, as indicated in the fourth experiment. The results in this and another insulin experiment suggest that each time the insulin is perfused there is a slight increase in the percentage of this protein filtered, and also there seems to be an increase in permeability toward egg

albumin. We have had to use a pH of 7 throughout the insulin experiments, since that is the upper limit of the pH stability range for insulin (7). This could possibly have influenced the results, but in experiments in which we have used much lower pH in perfusing other proteins, the filtrations were decreased rather

TABLE III  
*Indirect Experiments on Bullfrogs*

Experiment No.	Period No.	Protein perfused	Inulin urine to perfusion fluid ratio	Protein			
				Perfusion fluid	Urine	Glomerular filtrate	Per cent filtered
				per cent	per cent	per cent	
1	1	Hen's egg albumin	1.15	0.63	0.36	0.31	49
	2	Lactoglobulin	1.19	0.58	0.52	0.44	76
	3	Hen's egg albumin	1.16	0.63	0.39	0.34	54
	4	Lactoglobulin	1.17	0.58	0.54	0.46	79
2	1	Duck's egg albumin	1.21	0.64	0.44	0.36	56
	2	Hen's " "	1.10	0.58	0.33	0.30	52
	3	Lactoglobulin	1.15	0.47	0.42	0.37	79
	4	Duck's egg albumin	1.11	0.64	0.40	0.36	56
3	1	Hen's " "	1.30	0.130	0.095	0.073	56
	2	PPI	1.05	0.107	0.095	0.091	85
	3	Hen's egg albumin	1.10	0.130	0.080	0.073	56
4	1	Egg albumin	1.19	0.31	0.175	0.147	47
	2	Amorphous insulin	1.26	0.50	0.095	0.076	15
	3	Egg albumin	1.15	0.31	0.175	0.152	49
	4	Amorphous insulin	1.11	0.50	0.100	0.090	18
	5	Egg albumin	1.03	0.31	0.180	0.175	56
	6	Amorphous insulin	1.10	0.50	0.110	0.100	20
	7	Egg albumin	1.05	0.31	0.210	0.200	64
5	1	" " "	1.320	1.01	0.80	0.606	60
	2	" " + 7% urethane	0.995	1.00	0.84	0.844	84
	3	" " + 7% "	0.995	1.00	0.94	0.946	95
	4	" " + 7% "	0.997	1.00	0.97	0.973	97
	5	" " + 7% "	0.998	1.00	1.00	1.00	100

than increased. Insulin was the only protein studied (aside from the protamine, salmine, which was decidedly toxic) which appeared to affect the permeability of the glomerular membranes in these experiments.

Ethyl urethane in concentrations up to 5 per cent perfused in



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plain Ringer's solution between egg albumin perfusions or added to the albumin fluid itself did not alter permeability, but in a concentration of 7 per cent increased permeability gradually (see Experiment 5 of Table III) and in a concentration of 10 per cent increased it quickly and completely. With the higher concentrations of urethane, the membranes also became permeable to horse serum albumin, while normally, and in the presence of urethane under 5 per cent, they were practically impermeable to this protein. Whenever permeability was increased, there were also other signs of toxicity; the inulin urine to perfusion fluid ratio usually dropped to 1 or sometimes even slightly below 1.

Sodium glycocholate added to egg albumin perfusion fluid to make a concentration of 0.1 per cent resulted immediately in

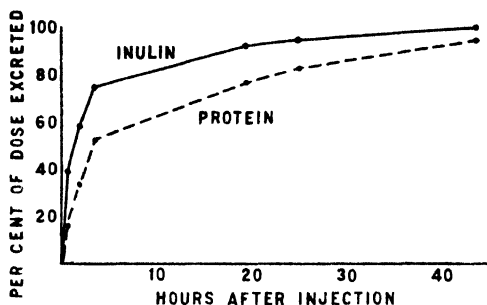


FIG. 3. Urinary excretion of injected inulin and egg albumin by the normal dog.

almost complete permeability to egg albumin. The same concentration of glycocholate also makes the membrane partially permeable to horse serum albumin. Concentrations of 0.02 per cent or below do not readily affect permeability. Contrary to expectations, the presence of 20 mg. per cent caffeine in an egg albumin perfusion fluid decreased the permeability slightly, as shown by the following percentage filtration: control 48, caffeine 42, control 47, caffeine 39, control 48. Cyanide in concentrations from 0.001 to 0.01 M had no marked effect on the filtration of egg albumin. Sometimes there was a slight increase and sometimes a decrease, both of which were within the range of experimental error.

*Excretion of Protein in Normal Dog*—Two unanesthetized dogs,

injected intravenously with 0.11 and 0.14 gm. of horse serum albumin per kilo of body weight, showed only traces of protein in the urine. Several dogs, injected with the same or larger amounts of egg albumin, promptly excreted protein in high concentration. The excretion of inulin and egg albumin after injection of about 3 gm. of the former and 3.4 gm. of the latter is graphically represented in Fig. 3. The cumulative excretion curves indicate that, although the protein is eventually excreted almost as completely as the inulin, the rate at which it is excreted is slower. There may be other explanations for this result but it certainly supports the belief that the glomeruli in this animal are considerably less permeable to egg albumin than to inulin. This would, of course, be in agreement with the results obtained on Amphibia.

#### DISCUSSION

The finding of practically complete impermeability of the glomerular membranes toward serum albumin, only partial permeability in the case of proteins with molecular weights approximately one-half that of serum albumin, and almost complete permeability in the case of the smallest protein (14,500 mol. wt.) agrees, on the whole, with the concept set forth in the introduction, that the passages in the membrane may be of various sizes, all of them being large enough to allow inulin to pass through with water but almost none of them large enough to allow the passage of horse serum albumin. We do not find, as is the impression gained from the statements of Bayliss, Kerridge, and Russell (23) concerning the mammalian kidney, that egg albumin is completely filtered. In other words, there appear to be some pores large enough for the passage of inulin but not of egg albumin. The observations nevertheless do not rule out the possibility that the pore size difference may be only "apparent" and that the results are due to some other characteristic of the membranes.

Since the diameter of the PPD-49609 has been found (9) to be approximately 20 Å., our data may be interpreted to mean that the mesh size over most of the glomerular membrane may be at least of this order. However, the difficulty which arises when we attempt to interpret the difference in filtrability between molecules supposedly of approximately the same size (especially egg albumin

and lactoglobulin) should serve to caution us in drawing too rigid conclusions and to remind us of the many complicating factors in the use of such test substances. We are mindful of the stimulating but complicating discoveries concerning the effects of other substances, of concentration, dilution, and even environment on the association and dissociation of the protein aggregates of which not only our test substances but possibly the membranes themselves are made (5-7). In an attempt to learn the condition of the egg albumin exactly as used experimentally, one portion of perfusion fluid containing this protein was subjected to ultracentrifugation with the result discussed earlier in the paper; the protein proved to be homogeneous, with a sedimentation constant characteristic of the pure protein. This is important evidence in support of the presumption that this protein has not been altered in any way so as to affect molecular weight by the procedures used, before it reaches the kidney. Moreover, there was no precipitation of egg albumin in perfusion fluid when the pH was brought back to the isoelectric point by the addition of acid. This and the easy crystallizability of egg albumin in these solutions when ammonium sulfate is added indicate that no denaturation, partial or complete, has taken place. Solutions oxygenated by pressure, a technique which provides less opportunity for surface denaturation,<sup>5</sup> behave similarly and show the same filtration. It seems relatively certain, then, that the egg albumin particles as used are unaltered. Molecular weights of proteins are usually given as dry weights but the weight for the hydrated egg albumin molecule has been calculated as 61,438 by Bull (26) and as 58,000 by Adair (27) who believes the molecule approximately spherical with a minimum radius of 26.4 Å. If these figures are accepted as correct, our data may be interpreted as signifying that the mesh size over about one-half of the glomerular membrane is at least 50 Å. in diameter, since the proteins as they exist in our perfusion fluids must be hydrated.

If egg albumin is taken as a standard, the filtration of lactoglobulin seems too high on the basis of weight or size. The wet molecular weight of lactoglobulin as determined from x-ray data by Crowfoot and Riley (see (6)) is 67,000 and the molecular volume

<sup>5</sup> Comparatively enormous pressures are required to denature proteins as was done by Bridgman (24) and Dow and Mathews (25).

88,000 cu. Å. Although we have no confirmatory molecular weight data on our own preparations of lactoglobulin, we have indication that the protein molecules have not been altered, in that the same results were obtained whether the solutions were aerated by pressure or by bubbling. Also, the protein did not precipitate out at the isoelectric point in salt solution. We have been unable, however, to recrystallize the lactoglobulin after it has been made up into perfusion fluid. Since the crystallization of this protein is not ordinarily as readily accomplished as is the case with egg albumin, this may not necessarily indicate that there has been some change in the lactoglobulin. The possibility of a partly denatured protein cannot be entirely ruled out, although it seems unlikely. Artificial membranes, prepared by treating No. 300 cellophane with zinc chloride (28) so that they became approximately 50 per cent permeable to egg albumin in perfusion fluid, were also 50 per cent permeable to lactoglobulin.<sup>6</sup> Since these membranes also allow the passage of a significant amount of serum albumin, it is probably true that they have a pore size distribution different from that of the glomerular membranes. However this may be, the suggestion remains that the glomerular membranes are in some way different from cellophane membranes which allow the passage of the same portion of egg albumin.

The sign and magnitude of the net charge of the protein molecule may be of importance in filtration through animal membranes. The work of Amberson and Klein (29), of Webster, Engel, Laug, and Amberson (30), of Ingraham and Visscher (31), and others has indicated that this may be a factor. The sign of the charge of the protein molecules used in our experiments must, with one exception, be negative, since the pH of the perfusion fluids is far above their isoelectric points. The exception is that of salmine, whose isoelectric point is so high that the particle bears a positive charge. The magnitude of the charges of the two proteins whose filtration differences are most puzzling to us (egg albumin and lactoglobulin) must be very nearly the same though perhaps not identical. Kekwick (32) has recently shown that in a mixture of

<sup>6</sup> We were privileged to obtain preliminary information concerning the filtrability of proteins through such membranes from Dr. W. B. Seymour of the Department of Medicine, Western Reserve University. The figures reported here are from our experiments.

0.55 per cent egg albumin and 0.51 per cent lactoglobulin in phosphate buffer at pH 8 and ionic strength of 0.1, "The mobilities of these two proteins are very close, under the conditions used, and the separation achieved is not sufficient to warrant an analysis of the curve into two simple curves corresponding to each component." It seems doubtful therefore, though not impossible, that the kidney could separate these two proteins because of differences in their net charges. Attempts were made to perfuse proteins at their isoelectric points and, although the conditions here were very unphysiological, a difference in filtration percentages persisted.

Mixtures of the two proteins gave percentage filtration figures intermediate between those of the two proteins perfused separately, discouraging the idea that either protein caused some change in the membrane which would make it more or less permeable.

One striking difference in the characteristics of the two proteins, egg albumin and lactoglobulin, is in the magnitude of their dipole moments (see (5) and (10) for references). A high dipole moment such as that of lactoglobulin may indicate either an elongated molecule or an unsymmetrical charge distribution. If the first explanation holds for this case, it might be possible for lactoglobulin molecules oriented properly to slip through pores which would not allow the passage of more spherical egg albumin molecules of approximately the same weight. If the second explanation holds, it might also be argued that a large mass of positively charged groups at one side of the particle with negatively charged groups at the other side could also help to orient the molecules with respect to the membrane pores if these pores carry a charge.

Again using egg albumin as the standard, we find the filtration percentage for insulin comparatively low. The ultracentrifuge studies (7) on insulin show dry molecular weights of 35,100 and 40,900. More recent x-ray studies by Crowfoot (6) give a dry molecular weight of 37,400, wet molecular weight of 52,400, and volume per "wet" molecule of 67,000 cu. Å. On the basis of size therefore, there is no reason to expect insulin to pass through the glomerular membranes less readily than egg albumin. The only dielectric dispersion data now available for insulin have been obtained in propylene glycol (5). We have, in short, no explanation for the comparatively low results with insulin but, as indicated earlier, our insulin experiments are open to the criticism that we

have little information concerning the dispersion of the protein in the solutions as used.

Recently several papers concerned with the excretion of hemoglobin have appeared in the literature (33, 34). Hemoglobin is excreted in the urine even though its molecular weight is regarded as near that of serum albumin. We have not used hemoglobin in any of our experiments as yet and do not believe that our results are necessarily in disagreement with these findings. Possibly the fact that this protein seems to be dissociated and associated rather readily (7) has not been given sufficient consideration. Although the concentrations of protein solutions and the conditions of our experiments may not be such that "athrocytosis" (34, 35) could be detected, we have not been able to demonstrate "re-absorption" due to this cause as is suggested by Monke and Yuile (34) for hemoglobin.

#### SUMMARY

1. A large number of direct (glomerular puncture) and indirect (protein-inulin) experiments have been performed on Amphibia to determine the extent of permeability of the glomerular membranes to small protein molecules.

2. Agreement between the results of the two methods is sufficiently good to warrant the belief that results obtained by the indirect method are not influenced by reabsorption of protein.

3. Although the glomerular membranes were found to be partially permeable to each of the proteins of the 35,200 molecular weight group, differences existed between the extents of this permeability toward the various proteins.

4. The purified protein derivative (mol. wt. 14,500) is filtered more completely than proteins of the group mentioned above.

5. In the light of present knowledge of the size of protein molecules it appears that most of the mesh surface of the glomerular membranes is coarse enough to permit the passage of particles about 20 Å. in diameter and that only one-half of it allows particles of about 50 Å. to go through.

6. Permeability of the membranes to proteins appears to be grossly according to size of particles, but there is a possibility that other factors influence the relative permeability toward particles of approximately the same size.

7. The indirect method provides an approach to measurement

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of effects on glomerular permeability. A few experiments illustrating this have been made with drugs and poisons.

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## A METHOD FOR THE DETERMINATION OF ADENOSINE-5'-PHOSPHORIC ACID AND ITS HOMOLOGUES\*

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For the determination of adenosine-5'-phosphoric acid and its homologues the methods which have been recommended chiefly are (1) enzymatic deamination by deaminase and quantitative determination of the ammonia split off (1); (2) determination on the basis of the pharmacological activity, (a) contraction of the isolated guinea pig uterus, (b) the characteristic change in the electrocardiogram upon intravenous injection into the rabbit (2); (3) isolation of the adenine by acid hydrolysis and nephelometric determination of its silver salt by comparison with standard solutions (3).

Each of these methods may have its advantages for the investigation of special problems. There exist, however, cases in which none of them yields satisfactory results, particularly if a high specificity is required and if only small amounts of adenylic acid are available. By methods (2) and (3) differentiation between adenosine-5'-phosphoric acid and adenosine-3'-phosphoric acid<sup>1</sup> is impossible; whereas, by method (1) adenosine as well as adenosine-5'-phosphoric acid is determined. Furthermore, in the latter method relatively large amounts are required, since the adenylic acid is the substrate of the enzymatic reaction. Despite the fact that the coenzyme properties of adenosine-5'-phosphoric

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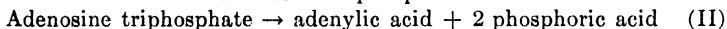
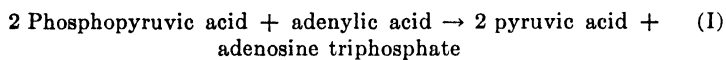
<sup>1</sup> For adenosine-5'-phosphoric acid and adenosine-3'-phosphoric acid the terms muscle adenylic acid and yeast adenylic acid respectively are used frequently; since these terms are misleading as far as the occurrence of these compounds is concerned, the chemical names are preferable.



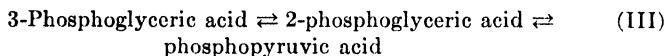
acid and its homologues, adenosine diphosphate and adenosine triphosphate, in several enzymatic systems have been known for a long time, no attempt has been made to utilize this property for their determination as has been done with other coenzymes, as, for example, the codehydrogenases and cocarboxylase.

A method is described here which had given such promising results in a preliminary form (4) that its systematic elaboration as a test reaction seemed to be desirable.

The principle of this method is the determination of adenosine-5'-phosphoric acid and its homologues by their coenzyme activity in the enzymatic cleavage of phosphoric acid from phosphopyruvic acid (5):



In the normal course of glycolysis the phosphoric acid is esterified with carbohydrate or creatine (6). In the absence of a suitable acceptor, however, free phosphoric acid appears, the amount of which under certain experimental conditions depends on the concentration of the adenosine-5'-phosphoric acid. Since phosphopyruvic acid is relatively difficult to obtain, *d*(-)-3-phosphoglyceric acid is used as a substrate. The enzyme preparations from muscle contain the enzymes necessary for the rearrangements (Reaction III) in such an amount that these reactions do not determine the rate of splitting of phosphopyruvic acid (5).



Pillai has studied the dephosphorylation of phosphopyruvate under various conditions (7). He claims that beside Reactions I and II another mechanism of dephosphorylation exists, since some muscle enzyme preparations do not contain adenylypyrophosphatase and therefore Reaction II seems to be ruled out under certain conditions. Nevertheless adenosine triphosphate is an essential part of this phosphoglycerate dephosphorylating system, according to Pillai. Therefore, the method to be described is independent of the mechanism which predominates.

The components of the test system are *d*(-)-3-phosphoglyceric acid as a substrate; as source of the enzymes, an extract of a dry

muscle preparation from which the adenosine-5'-phosphoric acids have been removed as completely as possible by dialysis;  $Mg^{++}$  as a necessary activator; and varying amounts of adenylic acid or the solution to be tested for coenzyme activity. After the incubation period (2 hours) the reaction is stopped by adding trichloroacetic acid and the amount of phosphoric acid split off is determined colorimetrically. The adenylic acid content of an unknown solution is determined by comparison with the coenzyme activity (phosphoric acid split off) of known amounts of adenylic acid in a series of parallel determinations, yielding a standard curve.

#### EXPERIMENTAL

*Enzyme Preparation*—All enzymes required for Reactions I to III are contained in dry muscle preparations, made according to Meyerhof (8). Rat or rabbit muscle is a suitable material for preparing the enzymes. Muscle tissue of the hind legs is taken immediately after the animal is sacrificed, carefully minced, and extracted at low temperature ( $0-2^{\circ}$ ), first with the same volume of 0.6 per cent potassium chloride solution, centrifuged, and resuspended in half the amount of potassium chloride solution. The mixture should be shaken vigorously each time for about 15 minutes. The opalescent supernatant fluids are combined and dialyzed at  $0-2^{\circ}$  in cellophane tubing against running distilled water, or a large volume of distilled water, which should be replaced several times. Mechanical stirring and addition of a few drops of toluene are advisable. Under these conditions 36 to 48 hours of dialysis were found to be sufficient to remove most of the adenylic acid. The contents of the cellophane tubing are centrifuged to remove inactive precipitate. The clear solution is precipitated with 10 volumes of cooled acetone. The precipitate is collected by centrifugation, washed with acetone, and dried *in vacuo*. The preparations thus obtained are active for months if stored in a desiccator.

For the experiment the very finely pulverized, dry muscle preparation is extracted with disodium barbital buffer (9) and to the extract the necessary amount of magnesium salt is added. For each ml. of enzyme solution 30 mg. of dry muscle preparation are taken up in 0.9 ml. of barbital buffer (5.54 ml. of 0.1 M disodium barbital + 4.46 ml. of 0.1 N HCl; pH 7.2) and 0.1 ml. of 0.1

$\text{M MgSO}_4$ . The mixture is stirred and shaken for about 1 hour at  $0^\circ$ , or half an hour at room temperature, centrifuged, and further clarified by filtration.

*Phosphoglyceric Acid*— $d(-)$ -3-Phosphoglyceric acid is easily obtained from fluoride-treated fermentation mixtures prepared according to Neuberg and coworkers (10). Glucose was used as a substrate instead of hexose diphosphate, since the latter is not a commercial product.

For the experiment the barium salt is treated with sodium sulfate to remove barium. For each ml. of solution 15 mg. of barium salt are treated with 0.45 ml. of  $0.1 \text{ M Na}_2\text{SO}_4$  solution + 0.35 ml. of  $0.1 \text{ N NaOH}$  + 0.2 ml. of  $\text{H}_2\text{O}$ . After shaking for  $\frac{1}{2}$  hour  $\text{BaSO}_4$  is removed by centrifugation.

*Composition of Samples*—The reaction mixture consists of 0.5 ml. of phosphoglyceric acid solution, 0.5 ml. of enzyme solution, adenylic acid solution, or the sample to be examined, and water to a total volume of 2.0 ml. Small centrifuge tubes or test-tubes are suitable. After 2 hours incubation at  $30^\circ$ , the reaction is stopped by addition of 3.0 ml. of 10 per cent trichloroacetic acid. After the mixture has stood for a short time in the cold, the protein precipitate is centrifuged off, and in an aliquot of the clear solution (1.0 ml.) free phosphate is determined by the Fiske and Subbarow method (11), with a photoelectric colorimeter. For comparison of the activating effect of the adenylic acid standard samples with that of the unknown solutions, the result is expressed in micrograms of P split off, per cent P split off, or simply as a galvanometer reading of the P determination.

*Coenzyme*—The adenosine-5'-monophosphoric acid used in these experiments was prepared from yeast (12) and was recrystallized repeatedly. Adenosine triphosphoric acid was obtained from rabbit muscle (13). The ratio of readily hydrolyzable phosphate to total phosphate was 1.8:3 (theoretical 2:3). The exact determination of the concentration of the coenzyme solutions in the experiment represented by Fig. 1 was carried out by determination of pentose according to the method of Bial as modified by Mejsbaum (14).

As can be seen from Fig. 1, adenosine triphosphate causes a somewhat higher dephosphorylation effect. The difference in comparison to adenylic acid corresponds approximately to the

amount of phosphoric acid required by the phosphorylation of adenylic acid to adenosine triphosphate (Reaction I). Since adenylic acid in aqueous solution is stable for a long time, if stored in the cold, its use for the comparison standard is recommended. It is unnecessary to neutralize the small amounts of coenzyme (10 to 100  $\gamma$ ), but it is important that the solutions to be examined should be adjusted to a correct pH value, since they frequently have a considerable buffering capacity. Furthermore, care should be taken that the salt concentration is low. Neutral salts in con-

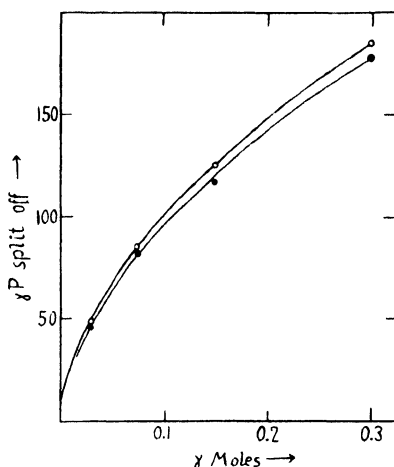


FIG. 1. Activation of the enzymatic splitting of phosphopyruvic acid by adenosine-5'-monophosphoric acid (●) and adenosine-5'-triphosphoric acid (○). The composition of the samples is given in the text.

centrations higher than 0.05 M have an inhibitory effect. Absence of free phosphate must be ascertained; otherwise a correction of the result obtained has to be made.

*Mg<sup>++</sup> and Mn<sup>++</sup> As Activator*—As Lohmann and Meyerhof have stated, Mg<sup>++</sup> is an essential activator of the phosphopyruvate dephosphorylating system (5). The influence of different amounts of Mg<sup>++</sup> on the reaction is apparent from Table I. Mn<sup>++</sup> can replace Mg<sup>++</sup> as activator. However, far greater amounts are required to obtain the maximum activation than are found in any animal tissues.

*Influence of pH*—The pH-activity curve is shown in Fig. 2.

The different pH values were obtained by barbital buffer solutions according to Michaelis (9). For the experiments in acid medium

TABLE I

*Activation of Enzymatic Splitting of Phosphopyruvic Acid by  $Mg^{++}$  and  $Mn^{++}$*

Experiment No.	Activator	Coenzyme (adenosine-5'-phosphoric acid)	P split off
		$\gamma$	$\gamma$
1			1.5
2	10 $\gamma$ $Mg^{++}$		12.5
3	50 $\gamma$ "		24.0
4	140 $\gamma$ "		27.0
5		25	4.0
6	10 $\gamma$ $Mg^{++}$	25	30.0
7	50 $\gamma$ "	25	53.0
8	140 $\gamma$ "	25	57.0
9	2 $\gamma$ $Mn^{++}$	25	25.0
10	10 $\gamma$ "	25	59.0
11	100 $\gamma$ "	25	74.5
12	2 $\gamma$ " + 140 $\gamma$ $Mg^{++}$	25	56.0
13	10 $\gamma$ " + 140 $\gamma$ "	25	57.0

The activator and coenzyme concentration are as given above in the complete sample (2.0 ml.).

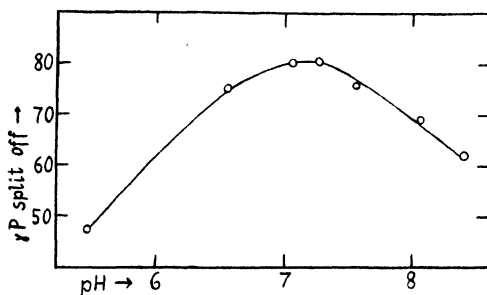


FIG. 2. pH-activity curve. The composition of the samples is given in the text.

the phosphoglyceric acid solution was only partially neutralized. The pH values as given in Fig. 2 are those of the complete system. The standard proportions were used, but the dry muscle

preparation was extracted with water and the buffer solutions added separately. 25  $\gamma$  of adenylic acid as coenzyme and 120  $\gamma$  of  $Mg^{++}$  as activator were added in each experiment.

*Incubation Time*—Fig. 3 shows the influence of the incubation time on the rate of dephosphorylation. 2 hours were found to be a suitable incubation time, since it provides a splitting of phosphopyruvate to such an extent that the effect of varied amounts of adenylic acid is distinct. A longer incubation period would cause a liberation of adenylic acid from labile nucleotides, as for example flavin-adenine dinucleotide and codehydrase I (4).

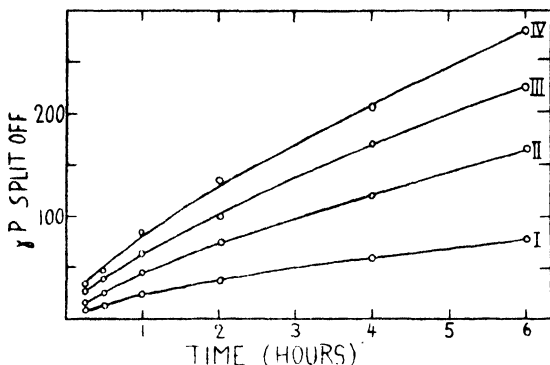


FIG. 3. Influence of the incubation time on the rate of dephosphorylation. The experimental conditions are given in the text. Curve I, blank experiment; Curve II, 20  $\gamma$  of adenylic acid per sample; Curve III, 50  $\gamma$  of adenylic acid per sample; Curve IV, 100  $\gamma$  of adenylic acid per sample.

*Blank Effect*—The enzyme preparations as a rule split phosphopyruvic acid to some extent without the addition of adenylic acid. This effect is probably due to the adenylic acid which has not been removed from the enzyme by the dialysis. Therefore, in each series a blank experiment should be made in order to obtain a correction. Depending on the nature of the enzyme preparation, the blank values range between 30 and 100  $\gamma$  of P. For the calculation a curve is drawn from the corrected values.

That the effect in the blank experiment is caused by adenylic acid present in the enzyme preparations is evident from the fact that a heat extract of enzyme, used as a coenzyme in the test system, activates the reaction in the expected magnitude. Deter-

minations of phosphate and of pentose (14) in the extracts from dry muscle preparations corroborate this suggestion. The residues

TABLE II  
*Specificity of Adenosine-5'-Phosphoric Acid As Coenzyme of  
Dephosphorylation of Phosphopyruvic Acid*

Experiment No.	Coenzyme	P split off	Adenosine-5'-phosphoric acid recovered
		$\gamma$	per cent
1		65	
2	10 $\gamma$ adenosine-5'-phosphoric acid	95	
3	25 $\gamma$ " "	115	
4	50 $\gamma$ " "	148	
5	100 $\gamma$ " "	185	
6	100 $\gamma$ adenosine-3'-phosphoric "	63	
7	300 $\gamma$ " "	68	
8	25 $\gamma$ adenosine-5'-phosphoric " + 100 $\gamma$ adenosine-3'-phosphoric acid	117	104
9	25 $\gamma$ adenosine-5'-phosphoric acid + 300 $\gamma$ adenosine-3'-phosphoric acid	119	108
10	30 $\gamma$ adenosine	72	
11	150 $\gamma$ "	72	
12	80 $\gamma$ adenosine-5'-phosphoric acid + 30 $\gamma$ adenosine	178	105
13	50 $\gamma$ adenosine-5'-phosphoric acid + 75 $\gamma$ adenosine	152	105
14	30 $\gamma$ inosinic acid	69	
15	90 $\gamma$ " "	69	
16	300 $\gamma$ " "	60	
17	50 $\gamma$ adenosine-5'-phosphoric acid + 150 $\gamma$ inosinic acid	141	92
18	20 $\gamma$ adenosine-5'-phosphoric acid + 240 $\gamma$ inosinic acid	108	95

of adenylic acid cannot be removed from the muscle preparations by extended dialysis without loss of enzymatic activity. It is possible that this part of adenosine-5'-phosphoric acid is bound in a more stable linkage to the protein.

### Results

*Coenzyme Specificity and Influence of Related Compounds*—Adenosine, adenosine-3'-phosphoric acid, and inosinic acid cannot replace the adenosine-5'-phosphoric acids as coenzyme (Table II). In additional experiments it was tested whether and in what magnitude these compounds have an influence on the coenzyme activity of adenosine-5'-phosphoric acid. Experiments 8, 9, 12, and 13 show that adenosine-3'-phosphoric acid and adenosine seem to have a slight activating effect upon adenosine-5'-phosphoric acid, whereas inosinic acid has some inhibitory effect (Experiments 17 and 18).

The results of these experiments are in agreement with the previous knowledge concerning the specificity of adenosine-5'-phosphoric acid and its homologues in this reaction.

*Some Examples for Use of Method*—The principle of this method was valuable in the examination of cozymase preparations previous to the elaboration of the regular test system described above. Pure cozymase does not act as a coenzyme of the splitting of phosphoglyceric acid (Table III, Experiments 6 to 8), whereas crude preparations contain notable amounts of adenosine-5'-phosphoric acids and therefore have some activity (Experiments 11, 12). Hydrolysis of cozymase in alkaline solution yields large quantities of adenosine-5'-phosphoric acid (Experiments 13, 14).

The examination of cozymase preparations for contamination by adenylic acid is one of the most important criteria in stating the degree of purity.

As was mentioned previously (15), adenylic acid is obtained not only by alkaline hydrolysis but also by acid hydrolysis of cozymase. The method herein described has now made it possible to determine the yield of adenylic acid by acid hydrolysis.

As can be seen from Table IV, very little adenosine-5'-phosphoric acid is formed by hydrolysis of cozymase in 0.1 N HCl, since adenine is split off almost as fast as the adenylic acid is formed.

Another application of the method was the examination of desaminocozymase. This cozymase derivative (16) contains, besides nicotinamide, hypoxanthine as a base, or inosinic acid instead of adenylic acid. There is some interest in the possibility of substituting desaminocozymase for cozymase as the vitamin,



TABLE III  
*Examination of Cozymase Preparations for Contamination by  
 Adenosine-5'-Phosphoric Acid*

Experi- ment No.	Coenzyme	P split off	Note
		$\gamma$	
1		36	
2	10 $\gamma$ adenosine-5'-phosphoric acid	95	
3	25 $\gamma$ adenosine-5'-phosphoric acid	127	
4	50 $\gamma$ adenosine-5'-phosphoric acid	156	
5	100 $\gamma$ adenosine-5'-phosphoric acid	197	
6	100 $\gamma$ cozymase (A)	44	<1% adenosine-5'-phosphoric acid
7	300 $\gamma$ " "	47	<1% adenosine-5'-phosphoric acid
8	1000 $\gamma$ " "	72	<1% adenosine-5'-phosphoric acid
9	25 $\gamma$ adenosine-5'-phosphoric acid + 100 $\gamma$ cozymase (A)	129	102% adenosine-5'-phosphoric acid recovered
10	50 $\gamma$ adenosine-5'-phosphoric acid + 250 $\gamma$ cozymase (A)	162	105% adenosine-5'-phosphoric acid recovered
11	100 $\gamma$ cozymase (B)	107	Adenosine-5'-phosphoric acid content found, 15%
12	250 $\gamma$ " "	131	Adenosine-5'-phosphoric acid content found, 11%
13	100 $\gamma$ " (A) hydrolyzed with alkali*	128	Adenosine-5'-phosphoric acid formed, 26%
14	300 $\gamma$ cozymase (A) hydrolyzed with alkali*	199	Adenosine-5'-phosphoric acid formed, 30%

Cozymase (A), highest purity; (B), a crude product of about 75 per cent cozymase activity.

\* 5.0 mg. of cozymase were dissolved in 0.5 ml. of  $H_2O$ , 0.5 ml. of 0.1 N NaOH was added, and the solution heated for 5 minutes in a boiling water bath. After cooling 0.5 ml. of 0.1 N  $H_2SO_4$  and water to a volume of 5.0 ml. were added. Of this solution 0.1 and 0.3 ml. were used for the adenosine-5'-phosphoric acid determination (Experiments 13 and 14).

TABLE IV

*Formation of Adenosine-5'-Phosphoric Acid from Cozymase by Hydrolysis in 0.1 N Acid*

Time of hydrolysis	Yield of adenosine-5'-phosphoric acid
min.	per cent
0	2
5	4
10	6
15	7
20	7
30	5
60	3

17.2 mg. of cozymase were dissolved in 10.0 ml. of 0.1 N  $\text{H}_2\text{SO}_4$ . Of this solution samples of 1.0 ml. each were heated in sealed tubes for different times. After cooling they were neutralized with 1.0 ml. of 0.1 N NaOH and water was added to a volume of 5.0 ml. Of this solution 1.0 ml. (corresponding to 344  $\gamma$  of cozymase) was used for the adenylic acid determination. Quantitative splitting into adenylic acid would give 52.3 per cent.

TABLE V

*Examination of Desaminocozymase*

Experiment No.	Coenzyme	P split off	Note
		$\gamma$	
1		44	
2	10 $\gamma$ adenosine-5'-phosphoric acid	64	
3	25 $\gamma$ adenosine-5'-phosphoric acid	87	
4	50 $\gamma$ adenosine-5'-phosphoric acid	116	
5	100 $\gamma$ adenosine-5'-phosphoric acid	147	
6	250 $\gamma$ desaminocozymase	43	
7	1250 $\gamma$ " "	42	
8	250 $\gamma$ " " hydrolyzed by alkali*	46	<1% adenosine-5'-phosphoric acid
9	1250 $\gamma$ desaminocozymase, hydrolyzed by alkali*	51	<1% adenosine-5'-phosphoric acid
10	20 $\gamma$ adenosine-5'-phosphoric acid + 1000 $\gamma$ desaminocozymase	84	23 $\gamma$ adenylic acid recovered

\* Alkaline hydrolysis of desaminocozymase was carried out as follows: 6.25 mg. of substance were dissolved in 1.0 ml. of 0.05 N NaOH, heated for 5 minutes in a boiling water bath, cooled, and neutralized with 1.0 ml. of 0.05 N HCl. Water was added to a volume of 5.0 ml.

since the latter has some undesirable properties due to its adenylic acid content (17). Desaminocozymase is prepared from cozymase by treatment with nitrous acid. The completeness of the deamination was tested by alkaline hydrolysis of the preparations and examination by the adenylic acid test method (Table V).

Whereas cozymase yields adenylic acid by alkaline hydrolysis (Experiments 13, 14, Table III), desaminocozymase yields inosinic acid, which is inactive.

Further work is in progress on the adaptation of the method of the determination of adenylic acid in blood and tissues.

#### SUMMARY

1. A method for the determination of small amounts of adenosine-5'-phosphoric acid and its homologues is described. This method is based on the coenzyme properties of these compounds in the enzymatic splitting of phosphopyruvic acid.

2. Some examples of the specificity of the method and of its application in nucleotide chemistry are described.

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## LETTERS TO THE EDITORS

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### A MODIFICATION OF THE SNELL AND STRONG MICROBIOLOGICAL METHOD FOR DETERMINING RIBOFLAVIN

*Sirs:*

In determining the riboflavin content of foodstuffs we have obtained excellent agreement between the Snell and Strong<sup>1</sup> microbiological method and the Hodson and Norris<sup>2</sup> fluorometric method except in the case of wheat products. When we attempted to analyze flour and flour enriched with riboflavin, higher results were obtained by the Snell and Strong procedure, in some cases amounting to 200 to 300 per cent recovery of the added riboflavin. Since flour contains a large quantity of starch, it occurred to us that the lactic acid-stimulating substance might be present in the starch and that it could be destroyed by enzymatic hydrolysis with taka-diastrase.

Hence suitable samples of wheat products were autoclaved in 250 ml. of 0.1 N HCl for 15 minutes at 15 pounds pressure. After the material was cooled to 50°, the pH was adjusted to 4 to 4.5 with NaOH and 1 gm. of taka-diastrase was added. The mixture was incubated overnight at 50° in order to hydrolyze the starch completely and then autoclaved for 5 minutes at 15 pounds pressure to inactivate the taka-diastrase. After the mixture was cooled to room temperature, the pH was adjusted to 6.6 to 6.8. The mixture was then made to a volume of 500 ml. and stirred in a Waring blender until a homogeneous suspension was obtained. Finally, suitable aliquots were transferred to the assay tubes and the remainder of the determination conducted in accordance with the Snell and Strong<sup>1</sup> procedure. The results of this study are presented in the accompanying table.

It is evident from the results obtained in these studies that starch, or some unknown substance in it, stimulates the production

<sup>1</sup> Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).

<sup>2</sup> Hodson, A. Z., and Norris, L. C., *J. Biol. Chem.*, **131**, 621 (1939).

Material	Method		
	Snell-Strong micro-biological	Taka-diastase modification of micro-biological	Hodson-Norris fluorometric
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
Patent flour.....			0.525
“ “ + 3.675 $\gamma$ riboflavin per gm.....	9.55	4.10	4.30
Bread from same flour.....	6.25	4.30	4.05
Whole wheat flour*.....	1.73	1.32	1.29
Red dog flour.....	4.45	2.65	2.80
Starch + 0.175 $\gamma$ riboflavin per gm.....	0.21	0.175	0.175

\* These results by Gillis, M. B., and Hill, F. W., Cornell University.

of lactic acid by *Lactobacillus casei*. In determination of the riboflavin content of foodstuffs containing starch, the lactic acid-stimulating properties can be destroyed by enzymatic hydrolysis with taka-diastase.

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Received for publication, August 15, 1941

## THE LIVER CATALASE ACTIVITY OF TUMOR-BEARING RATS AND THE EFFECT OF EXTIRPATION OF THE TUMORS

*Sirs:*

We have shown<sup>1</sup> that the liver catalase activity of rats carrying the subcutaneously implanted hepatic tumor No. 31<sup>2</sup> was about one-tenth that of the livers of normal rats. In order to determine whether this lowering of liver catalase activity was directly due to the presence of the transplanted tumor, the latter was completely excised from a number of animals under ether anesthesia. About half the number of these animals was sacrificed at intervals and the liver catalase activity determined. The remainder of the animals, 48 hours after operation, were reinoculated subcutaneously with fresh tumor material, and the second tumor allowed to grow for 4 weeks. The liver catalase at this point was determined on a few animals, and from the remainder the tumors were again excised. The latter animals were sacrificed at intervals following this second operation and the liver catalase activity determined. The data are given in the accompanying table. The method of determining the catalase activity has been described.<sup>1</sup> Included in the table are data on regenerating rat livers.

The data reveal (a) that the lowered liver catalase activity of tumor-bearing rats is attributable to the presence of the tumor, (b) that this effect is apparently reversible, and (c) that liver regenerating in animals carrying the tumor behaves like non-regenerating liver under the same circumstances.

Further experiments have shown that fasting has no apparent effect on the liver catalase activity of either normal or tumor-bearing rats.

<sup>1</sup> Greenstein, J. P., Jenrette, W. V., and White, J., *J. Nat. Cancer Inst.*, **2**, in press (1941).

<sup>2</sup> Greenstein, J. P., Jenrette, W. V., Mider, G. B., and White, J., *J. Nat. Cancer Inst.*, **1**, 687 (1941).



*Rat Liver Catalase Activity*

The tissue extracts contained 0.6 mg. of N per cc.

Tissue	Remarks*	Evolution of oxygen
		cc. per sec.
1. Normal rat liver	Animals 5 wks. to 1 yr. old	1.7
2. Regenerating liver in normal rats	" 5 " " 1 " "	1.6
3. Livers of rats carrying transplanted hepatic tumor No. 31	Tumors 4 wks. old; host rats 9-10 wks. old	0.1
4. Livers of rats from which transplanted tumors were removed	24 hrs. after operation	1.0
	48 " " "	1.8
	72 " " "	1.8
	96 " " "	1.3
	168 " " "	1.8
5. Rats described under (4) inoculated with fresh tumor tissue	Second tumors 4 wks. old; host rats about 14 wks. old	0.1
6. Rats described under (5) from which second tumor was removed	24 hrs. after operation	0.9
	48 " " "	1.8
	72 " " "	1.8
7. Regenerating liver in animals carrying transplanted hepatic tumors 4 wks. old	24 " " removal of lobes	0.3
	48 " " " " "	0.3
	72 " " " " "	0.1
	120 " " " " "	0.1
8. Rats described under (7) from which tumors were removed	24 hrs. after removal of tumor, 6 days after removal of lobes	1.0
	48 hrs. after removal of tumor, 7 days after removal of lobes	1.6
	72 hrs. after removal of tumor, 8 days after removal of lobes	1.8

\* Age of tumor refers to the time elapsed since implantation.

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Received for publication, September 10, 1941

## STEROIDS

### IV. THE FATE IN MAN OF INJECTED $\alpha$ -ESTRADIOL\*

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(Received for publication, July 28, 1941)

Since the establishment of  $\alpha$ -estradiol as the principal estrogenic substance of follicular fluid (Doisy and coworkers (2)), the view has been generally held that the less potent urinary estrogens, estrone and estriol, represent excretory transformation products of this primary ovarian hormone. The obvious test of the validity of the hypothesis is the ability to demonstrate such a conversion in the animal body. Biological and colorimetric assays of fractionated extracts of urine from the  $\alpha$ -estradiol-injected monkey (Westerfeld and Doisy (3)) and rabbit (Pincus (4)) have indicated the ketonic and weakly phenolic character of a large proportion of the estrogens excreted, and thus good evidence of dehydrogenation of  $\alpha$ -estradiol to estrone in these species is provided. While this reaction proceeds in the absence of uterus and ovary (3, 4), the possible conversion to estriol, as judged by increase of activity in the strongly phenolic urine fraction and its positive David (5) color test, is dependent upon the presence of a functional uterus (Pincus and Zahl (6), Pincus (4)). More recently, chemical proof of the oxidation of  $\alpha$ -estradiol to estrone in the ovariectomized guinea pig has been achieved with the isolation of the latter after administration of the former (Fish and Dorfman (7)). The examination by strictly chemical methods of the fate of  $\alpha$ -estradiol in man herein reported was undertaken to gain insight into the interconvertibility of the various estrogens in the human and to elucidate other aspects of estrogen metabolism, presently discussed.

\* Presented in part before the American Society of Biological Chemists, 1941 (1).

A total of 250 mg. of carefully purified  $\alpha$ -estradiol in 30 ml. of corn oil was given intramuscularly over a period of 8 days to a normal male subject. The patient experienced moderate nausea which ceased abruptly on discontinuance of injections. Gynecomastic changes were manifested from the 2nd day by symmetrical hypertrophy of the breasts and pronounced tenderness of nipples and areoli; regression was gradual over several weeks, presumably because of slow absorption of residual hormone. The urine excreted during the administration period and the following 10 days was strongly acidified (40 ml. of concentrated hydrochloric acid per liter) and autoclaved at 15 pounds pressure for 2 hours, which treatment effects optimum hydrolysis of the conjugated estrogens in normal male human urine (Callow, Callow, Emmens, and Stroud (8)). After extraction with benzene and removal of acids with sodium carbonate, 890 mg. of phenols and 957 mg. of neutral substances were obtained. The ketonic phenols (40 mg.), separated with Girard's (9) Reagent P, gave up on sublimation 16.2 mg. of estrone, which was identified by mixed melting point determination and as benzoate. The extent of the conversion of  $\alpha$ -estradiol to estrone in man is thus 6.4 per cent; Fish and Dorfman (7) obtained 3.1 to 3.7 per cent in the guinea pig. From the non-ketonic phenols (113 mg. after removal of light oils distillable below 115°), no unchanged  $\alpha$ -estradiol could be precipitated with digitonin, but, by adsorption on aluminum oxide and elution with 10 per cent acetone in ligroin, 9.8 mg. (3.9 per cent) were recovered. Careful examination of the remaining non-ketonic phenols by chromatographic separation into 63 fractions failed to reveal the presence of estriol<sup>1</sup> or of  $\beta$ -estradiol, a metabolite which Stroud (12) and Fish and Dorfman (13) isolated from the urine of the intact female or hysterectomized-ovariectomized rabbit following the administration of either  $\alpha$ -estradiol or estrone. Eluted with absolute ligroin was a small quantity (about 6 mg.) of an unidentified compound as prismatic crystals which melted at 198–202° and exhibited with sulfuric acid an intense greenish blue

<sup>1</sup> In view of the findings of Pincus (4, 6), it is improbable that estriol would be formed in the absence of a uterus. Also previous treatment excludes the presence of more than residual traces of estriol at this stage, as it is almost completely extractable from benzene with carbonate (Mather (10); Bachman and Pettit (11)).

fluorescence. The substance proved to be non-phenolic, as judged by its insolubility in aqueous alkali and negative Millon and xanthoproteic reactions. Salt formation readily took place in warm ethanolic sodium hydroxide solution, which accounts for the presence of the compound in the "phenolic" fraction and possibly indicates the opening of a lactone ring.

Only a small proportion of the hormone injected is thus accounted for as phenolic estrogen (10.3 per cent, based on the quantity of estrone and unchanged  $\alpha$ -estradiol isolated). This is entirely consistent with the findings of many investigators concerning the fate of estrogens in the body (for reviews of the literature, see Allen, Hisaw, and Gardner (14) and Zondek (15)). Irrespective of species or of the chemical nature of the estrogen given, no more than 3 to 20 per cent of the biological activity of the hormone administered is demonstrable in the urine. While much earlier work in this connection is largely invalidated because of improper hydrolytic treatment, the low recovery is amply borne out by the results of more recent and the present investigations in which optimal conditions of hydrolysis and efficient methods of extraction are employed. Since the quantity of estrogen eliminated by way of the intestine, or stored in the organism, is negligible (Zondek (15), Dingemanse and Laqueur (16)), the conclusion is inescapable that the remaining 90 odd per cent undergoes chemical alteration (beyond simple conjugation) in such a manner that physiological potency is destroyed. Offered in explanation have been several reactions leading to products with little or no biological activity: (a) oxidative attack in the ortho or para position with respect to the phenolic hydroxyl group, analogous to the oxidation of tyrosine by tyrosinase (Raper (17)), suggested by Westerfeld (18) in view of the rapid inactivation *in vitro* of the natural estrogens by a thermolabile, cyanide-sensitive enzyme of liver (see Zondek (15), Heller (19)) and by the direct oxidases (laccase) of the mushroom (Westerfeld (18), Graubard and Pincus (20)) and other plants (15, 20); (b) rupture of Ring D at the carbonyl group, accomplished *in vitro* by oxidation of estrone in alkaline medium with hydrogen peroxide, with the formation of the corresponding  $C_{13}||C_{17}$  hydroxy acid, which lactonizes immediately on acidification of the alkaline solution (Westerfeld (21)). Possible substantiation of such a mechanism of inactivation of steroidal hormones in the body is

afforded by the excretion of similar lactones during pregnancy in the mare. A compound,  $C_{19}H_{26}O_3$ , previously isolated by one of us (Heard (22)), has been positively identified as a ketolactone (unpublished observations), and Jacobs and Laqueur (23) have encountered a closely related ketolactone with the same empirical formula; (c) complete saturation of the benzenoid ring to one or more of the corresponding estranediols. Two of these are constituents of the urine of normal women (Marker, Rohrmann, Lawson, and Wittle (24)), and the only logical presumption is that they arise from endogenous estrogen. Also the occurrence of  $\Delta$ -5,7,9-estratrien-3( $\beta$ )-ol-17-one in equine pregnancy urine (Heard and Hoffman (25)) strongly suggests that reduction of the benzenoid Ring A of equilenin proceeds *in vivo*.

Mindful of these possible metabolic pathways, we conducted a thorough search for respective excretory products. Westerfeld's lactone (m.p. 335–340°) would appear in the phenolic fraction,<sup>2</sup> and it has already been mentioned that only  $\alpha$ -estradiol and an unidentified compound (m.p. 198–202°) were obtained therefrom. While the latter substance has certain lactonic properties, it is also non-benzenoid, and therefore not derived from  $\alpha$ -estradiol unless reduction in Ring A has taken place, but even so, the quantity excreted accounts for only 2.4 per cent of the hormone administered. Fully reduced estrane derivatives are neutral in reaction. Accordingly, the neutral fraction (957 mg.) was processed with Girard's reagent to give 217 mg. of ketones, which were separated into twenty-nine fractions by elution from alumina with carbon tetrachloride and carbon tetrachloride containing increasing quantities of ethanol (Callow's (26) procedure). Isolated in the approximate yield (mg. per liter of urine) indicated and identified by mixed melting point determinations were androsterone (0.76), dehydro-

<sup>2</sup> We have confirmed this partition between benzene, carbonate, and alkali. Excess of Westerfeld's lactone was shaken with 200 ml. of benzene, and, after filtration, the benzene solution was extracted three times with 10 per cent carbonate, and then with *N* sodium hydroxide solution. Acidification of the carbonate washings (acid fraction) yielded nothing, while from the sodium hydroxide extracts (phenolic fraction) was recovered the original lactone melting at 315–320° (uncorrected; long stem thermometer). Our thanks are due Dr. W. W. Westerfeld, who kindly furnished details of the preparation of the lactone from estrone.

isoandrosterone (0.47), and etiocholan-3( $\alpha$ )-ol-17-one (0.88). From the non-ketonic neutral fraction (540 mg.) 120 mg. of alcohols were removed by way of their half succinates, and divided with digitonin into an  $\alpha$  (104 mg.) and a  $\beta$  (15 mg.) portion. Chromatographic separation, respectively into 57 and twelve fractions, effected the isolation of pregnane-3( $\alpha$ ),20( $\alpha$ )-diol (0.17 mg. per liter) and cholesterol (0.07 mg. per liter). Concerning these five steroids, no further comment need be made, as each has been established as a normal constituent of male urine in this approximate amount by two or more independent groups of investigators (for a review of the literature, see Engel, Thorn, and Lewis (27)). Neither of the two estranediols or estranediones characterized by Marker, Rohrmann, Lawson, and Wittle (24) was encountered, nor any appreciable quantity of unidentified compounds which might conceivably represent other products of reduction of the estratriene nucleus (*i.e.*, the estranolones, and the isomeric estranediols and estranediones, all undescribed). In the latter connection, only two unknown substances were obtained, both from the non-ketonic  $\alpha$ -carbinol fraction, in traces only (respectively 4 and 3 mg.) and in crude state (m.p. 152–162° and 132–152°).

While it is realized that failure to isolate Westerfeld's lactone or any recognized estrane derivative does not afford conclusive proof of the total absence of reactions (b) and (c) in the male organism, it is quite apparent that an insignificant proportion, if any, of the injected  $\alpha$ -estradiol is metabolized by these routes. Conceivably dibasic acids, or hydroxy acids which lactonize only with difficulty, might arise on ring opening, and these would be contained in the unexplored carbonate extracts, but the burden of our evidence, albeit negative, credits the belief that the major part of the hormone is inactivated by an oxidative mechanism such as (a). Presumably this takes place in the liver, which is the only body tissue with marked power of inactivation of estrogen *in vitro* (15, 19). Also this site is indicated by the great loss of activity on perfusion of estrone through the surviving heart-lung-liver preparation in the dog, in contrast to relative stability in the heart-lung system (Israel, Meranze, and Johnston (28)), and by the increased effectiveness of estrogen in animals with livers poisoned with carbon tetrachloride (Talbot (29), Pincus and Martin (30)).

## EXPERIMENTAL

Melting points were observed under a cover-slip on a microscope slide heated on the stage of the Kofler-Hilbck micro melting point apparatus (31). The values recorded are corrected, the limits defining the temperature of first appearance of liquid and that of complete clearing of the melt.

Microanalyses were kindly carried out by Mrs. Dorothy Jewitt, Ayerst, McKenna and Harrison, Ltd., Montreal.

*Administration of  $\alpha$ -Estradiol and Processing of Urine*—The  $\alpha$ -estradiol used was prepared from estrone<sup>3</sup> by reduction with Raney's nickel in alkaline solution (method of Whitman, Wintersteiner, and Schwenk (32)). The product was purified via its digitonide and repeated recrystallization from ethanol and acetone to a constant melting point of 175–177°. 250 mg., in 30 ml. of corn oil (Mazola), were injected over 8 days into the deltoid muscles of one of us (M. M. H.). Urine was collected throughout the administration period and the succeeding 10 days (total volume 18.7 liters). With the accumulation of about 3 liters every 3rd day, concentrated hydrochloric acid was added (40 ml. per liter), and the mixture was autoclaved at 120° for 2 hours. On cooling, the solution was further acidified with concentrated hydrochloric acid (10 ml. per liter), approximately saturated with sodium chloride, and extracted ten times with 500 ml. portions of benzene. The combined benzene extracts of the entire output were concentrated *in vacuo* to 3 liters and washed free of acids with 10 per cent sodium carbonate solution (6  $\times$  350 ml.). Phenols were then shaken out with 1 N sodium hydroxide solution (10  $\times$  300 ml.) and collected from the acidified alkaline extracts with ether (5  $\times$  400 ml.); evaporation of the washed ethers yielded 890 mg. of semicrystalline reddish gum (Fraction A). The original benzene extract containing

<sup>3</sup> Generously provided by the Connaught Laboratories, University of Toronto, through the courtesy of Professor C. H. Best.

<sup>4</sup> In the Kofler-Hilbck apparatus (but not by the usual capillary method) pure  $\alpha$ -estradiol crystallized from ethanol or acetone melts partly in the neighborhood of 100°, resolidifies, and melts completely at 175–177°. As material sublimed *in vacuo* does not exhibit the double melting point, the phenomenon is probably due to solvent of crystallization, which can only be removed by vacuum desiccation at high temperature (David, de Jongh, and Laqueur (33)).

the neutral fraction was washed with water and taken to dryness (957 mg. of pigmented oil) (Fraction B).

*Isolation of Estrone*—The total phenols (Fraction A) in 8.5 ml. of absolute ethanol and 1.3 ml. of glacial acetic acid, were refluxed (1 hour) with Girard's (9) Reagent P (1 gm.). The solution was poured into an ice-water mixture (80 ml.) containing sufficient standard alkali to neutralize the acetic acid used, and then extracted four times with ether. Evaporation of the washed ethereal extracts yielded the non-ketonic phenolic fraction (C). The aqueous phase, together with the first water washing from Fraction C, was acidified with hydrochloric acid to 1 N and after 1 hour extracted four times with ether. On distillation of the solvent, 40.4 mg. of semisolid ketonic phenols resulted. These were subjected to distillation at approximately 0.01 mm. pressure; up to 115°, 7.7 mg. of light oils were removed, and from 170° to 180°, 16.2 mg. of crystals (m.p. 248–253°) sublimed. One crystallization of the latter from 95 per cent ethanol gave estrone melting at 252–255°, and at 254–257° on admixture with an authentic specimen (m.p. 257–260°).

For further identification, 10 mg. of the isolated estrone were benzoylated by the Schotten-Baumann method. After three recrystallizations from aqueous ethanol, the product (4.1 mg.) melted at 211–213°; mixed with authentic estrone benzoate (m.p. 213–215°), the melting point was 212–214°.

*Analysis*— $C_{18}H_{21}O(OCOC_6H_5)$ . Calculated. C 80.17, H 7.00  
Found. " 79.84, " 7.28

*Examination of Non-Ketonic Phenolic Fraction (Isolation of Unidentified Compound Melting at 198–202° and of  $\alpha$ -Estradiol)*—The non-ketonic phenols (Fraction C), which weighed 113 mg. after removal *in vacuo* of light oils distillable below 115°, were treated with digitonin (150 mg. in 7.5 ml. of 80 per cent ethanol) in an effort to separate the sparingly soluble digitonide of  $\alpha$ -estradiol. No precipitate formed on standing for 2 days. Ether (250 ml.) was then added, and the precipitated digitonin was collected and well washed with ether. After distillation of the solvent from the combined ethereal filtrate and washings, the residual oil was taken up in acetone (1.5 ml.), brought almost to the point of incipient precipitation by addition of ligroin (5 ml.), and adsorbed



on a column ( $8.5 \times 5$  cm.) of aluminum oxide (E. Merck, Darmstadt,<sup>5</sup> standardized according to Brockmann; 4 gm.) which had previously been saturated with ligroin. Elutions were carried out with successive 25 ml. portions of solvent, as indicated in Table I.

On evaporation of the ligroin from Fractions 2, 3, and 4, 6 mg. of solid material melting from  $170$ – $200^\circ$  remained. This was sub-

TABLE I  
*Chromatographic Separation of Non-Ketonic Phenolic Fraction*

Fraction No.	Eluent	Eluate			Compounds isolated
		Yield	Character	M.p.	
		mg.		$^\circ\text{C.}$	
1	Ligroin	1.6	Oil		
2-4	"	6.0	Crystalline	170-200	Unidentified, m.p. 198-202°
5-8	"	2.1	Gums		
9-12	Acetone(5%)-ligroin	7.4	"		
13	Acetone(10%)-ligroin	7.7	Semicrystalline		
14-17	Same	9.8	Crystalline	163-174	$\alpha$ -Estradiol
18	"	1.3	Gum		
19-22	Acetone(20%)-ligroin	15.8	Gums		
23-41	Acetone(30-100%)-ligroin	13.3	"		
42-60	Ethanol(10-100%)-acetone	30.1	"		
61-63	Ether	1.5	"		
Total.....		96.6			

limed at  $160^\circ$  and 0.01 mm., which procedure separated from a trace of oil about 4 mg. of large colorless prisms (m. p.  $198$ – $202^\circ$ ), very soluble in chloroform, sparingly so in ethanol. With sulfuric

<sup>5</sup> It is worth while calling attention to the observation of Talbot, Wolfe, MacLachlan, and Berman (34) that Merck's (Rahway) alumina, also standardized according to Brockmann, possesses different adsorptive properties with respect to certain steroids investigated by them. Consequently the conditions of adsorption and elution of the various compounds herein described need not obtain with the American product.

acid in the cold, the compound exhibited an intense greenish blue fluorescence, indistinguishable from that shown by  $\beta$ -estradiol (32). As evidence of the non-phenolic nature of the substance, Millon's test (modified method of Cole (35))<sup>6</sup> and the xanthoproteic reaction were negative, and a trace of the material, precipitated in finely divided state from ethanol with water, could not be dissolved in 1 N aqueous sodium hydroxide even on prolonged boiling. From solution in 0.5 N ethanolic sodium hydroxide, readily effected with the aid of heat, no precipitate was obtained on dilution with water, but acidification immediately caused flocculation. Judged from this behavior with alkali, it is probable that the compound is a lactone, but paucity of material prevented further characterization.

Fractions 14 to 17, eluted with 10 per cent of acetone in ligroin, afforded a total of 9.8 mg. of crystalline residue melting at 163–174°, with needles which sublimed on the cover-slip, at 170–174°. After purification by distillation (140°, 0.01 mm.), recrystallization from ethanol, and another sublimation, 2.1 mg. of stout prisms were obtained which melted at 175–177°, both alone and on admixture with authentic  $\alpha$ -estradiol.

Attempts to crystallize all of the remaining eluates from various solvents proved abortive.

*Examination of Ketonic Neutral Fraction (Isolation of Dehydroisoandrosterone, Androsterone, and Etiocholan-3( $\alpha$ )-Ol-17-One)*—The total neutral material (Fraction B) was first divided with Girard's Reagent P (500 mg.), as described above, into a ketonic (217 mg.) and a non-ketonic (540 mg.) (Fraction D) portion. The ketonic oil, in 5 ml. of carbon tetrachloride, was put through a chromatograph column (11  $\times$  0.8 cm.) containing 5 gm. of aluminum oxide previously saturated with carbon tetrachloride. Elutions with successive 25 ml. portions of eluent proceeded in accordance with Table II.

Fractions 5 to 8, eluted with absolute carbon tetrachloride, and carbon tetrachloride-ethanol (0.1 per cent), yielded semicrystalline

<sup>6</sup> In our experience, Millon's reagent prepared from mercury in the usual way is most unsatisfactory for detection of phenolic steroids. Only a yellow, or at best, a transient red coloration develops. In the modified mercuric-nitrite reaction of Cole, a minute quantity of estrone gives rise to an intense and fairly permanent orange-red solution.

solids melting over a wide range and containing considerable digitonin-precipitable material. They were therefore combined (total weight 29 mg.), and dissolved in 6 ml. of warm 90 per cent ethanol containing 75 mg. of digitonin. The digitonide precipitated after several days at  $-2^{\circ}$  was collected, washed with cold ethanol, and dried (55.5 mg.). Two decompositions in pyridine (Bergmann's procedure (36)) yielded 8.8 mg. of sticky crystals which were benzoylated in anhydrous pyridine (8 drops) with 4 drops of benzoyl chloride (overnight at room temperature). The

TABLE II  
*Chromatographic Separation of Neutral Ketonic Fraction*

Frac- tion No.	Eluent	Eluate			Compounds isolated
		Yield	Character	M.p.	
		mg.		$^{\circ}\text{C.}$	
1-4	$\text{CCl}_4$	61.5	Gums		Androsterone and dehydro- isoandros- terone
5	"	2.7	Crystalline	112-127	
6	Ethanol(0.1%)- $\text{CCl}_4$	2.4	"	108-136	
7-8	Same	23.9	Semicrystal- line	98-138	
9-11	"	35.0	Gums		Etiocholan- 3( $\alpha$ )-ol-17- one
12-14	"	16.4	Semicrystal- line	100-137	
15-18	Ethanol(0.2%)- $\text{CCl}_4$	3.2	Gums		
19-27	Ethanol(0.3-5%)- $\text{CCl}_4$	24.3	"		
28-29	Ethanol	24.6	"		
Total.....		193.7			

product was isolated with ether and twice recrystallized from acetone to give 2 mg. of *dehydroisoandrosterone benzoate* melting at  $240-247^{\circ}$ , and at  $243-250^{\circ}$  when mixed with an authentic specimen (m.p.  $253-254^{\circ}$ ).

The digitonin-non-precipitable part of Fractions 5 to 8, recovered on evaporation of the ethanolic filtrate and washings from the above digitonide after removal of excess digitonin by precipitation with ether, consisted of 20 mg. of amorphous solid, which was refluxed (3 hours) with 50 mg. of hydroxylamine hydrochloride in ethanol-water (3 ml. to 1 ml.) containing 10 mg. of sodium acetate. A

trace of flocculent material, insoluble in the hot solution, was discarded, and the oxime, filtered off after dilution with water, was crystallized from acetone (15 mg. of plates arranged in rosettes). It melted at 202–208° (sublimed crystals, 205–208°), and at 205–209° on admixture with *androsterone oxime* (m.p. 205–209°). The oxime was hydrolyzed (2 hours on the bath) with 1 ml. of 3 N sulfuric acid in 3 ml. of ethanol, and the *androsterone*, isolated with ether, had after sublimation at 130–140° (0.01 mm.) and two recrystallizations from aqueous methanol a melting point of 180–182° (unchanged on admixture with international standard androsterone, m.p. 183–184°).

Fractions 12 to 14, also eluted with 0.1 per cent ethanol in carbon tetrachloride, each consisted of a gummy solid, m.p. 100–137°, with sublimed out needles on the cover-slip, m.p. 135–137°. The three residues were combined (16.4 mg.) and distilled (135–140°, 0.01 mm.). Crystallization of the distillate from aqueous methanol deposited long slender needles, m.p. 144–148°, which melted at 145–148° when mixed with *etiocholan-3(α)-ol-17-one*<sup>7</sup> (m.p. 146–149°).

None of the remaining eluates could be induced to crystallize.

*Examination of Non-Ketonic Neutral Alcohols (Isolation of Cholesterol and Pregnane-3(α),20(α)-Diol)*—The non-ketonic neutral fraction (D), in 10 ml. of dry benzene and 2 ml. of anhydrous pyridine, was refluxed 1½ hours with 900 mg. of succinic anhydride. Ether (80 ml.) was added to the cooled solution, and pyridine removed by repeated washing with 2 N hydrochloric acid. The half succinates of the alcohols were then extracted with 10 per cent sodium carbonate (4 × 20 ml.) and collected from the acidified carbonate solutions with ether (4 × 40 ml.). Saponification of the residue from the washed ethers was effected by refluxing in 5 ml. of 3.5 N ethanolic potassium hydroxide for 1½ hours, when water (50 ml.) was added and the free alcohols were extracted with ether (4 × 20 ml.). On distillation of the solvent, 120 mg. of a semisolid orange gum remained. This was treated with digitonin (210 mg. in 14 ml. of 80 per cent ethanol), and, after 24 hours at –2°, the insoluble digitonides were collected, washed with cold ethanol, dried (83.4

<sup>7</sup> We are indebted to Dr. L. L. Engel for the specimen of *etiocholan-3(α)-ol-17-one*.

mg.), and twice decomposed in pyridine (36) to give 15 mg. of partly crystalline  $\beta$ -carbinols. The  $\alpha$ -carbinols, 104 mg. of an orange gum, were recovered from the ethanolic mother liquors as before.

The non-ketonic  $\beta$ -carbinols (15 mg.), in acetone-ligroin (0.3 ml. to 1 ml.), were adsorbed on a chromatograph column ( $6 \times 0.6$  cm.; 1.5 gm. of aluminum oxide) prepared with ligroin. Twelve elutions were made with successive 5 ml. portions of ligroin and acetone-ligroin mixtures in which the concentration of acetone was progressively raised. Only one eluate could be crystallized (Fraction 6, eluted with 5 per cent acetone in ligroin; 3.4 mg.); from aqueous ethanol, 1.3 mg. of platelets separated, and these melted at  $147-148^\circ$ , alone and on admixture with authentic *cholesterol*.

Likewise the digitonin-non-precipitable, non-ketonic  $\alpha$ -carbinols (104 mg.) were chromatographed. Dissolved in benzene-ligroin (0.5 ml. to 0.6 ml.) they were adsorbed on 3.5 gm. of aluminum oxide in a  $10 \times 0.8$  cm. column, and divided into thirty-two fractions with successive 25 ml. portions of ligroin, benzene-ligroin mixtures in which the concentration of benzene was gradually increased to 100 per cent, followed by progressively increasing proportions of acetone in benzene. Only Fraction 25 (14.3 mg.), dissolved out with 20 per cent acetone in benzene, contained solid material. Crystallized from aqueous acetone, it yielded 2 mg. of glistening platelets melting at  $210-236^\circ$  (large hexagonal plates, typical of pregnanediol, sublimed on the cover-slip and melted at  $231-236^\circ$ ). A trace of oil was distilled off below  $150^\circ$  in the Kofler micro vacuum bell (0.01 mm.); the main body of material sublimed around  $200^\circ$  and melted sharply at  $238-240^\circ$ , showing no depression when mixed with authentic *pregnane-3(\alpha),-20(\alpha)-diol* (m.p.  $237-239^\circ$ ). The following fraction, No. 26 (43.4 mg.), also eluted with 20 per cent of acetone in benzene, failed to crystallize. Since it contained a relatively large part of the total  $\alpha$ -carbinols, it was readsorbed on 1.4 gm. of aluminum oxide in a  $6 \times 0.6$  cm. column, and subdivided into twenty-five fractions with acetone-ligroin mixtures in which the acetone content was more gradually raised. Only two of these subfractions showed promise of yielding workable material. Fraction 5 (4.6 mg.), eluted with acetone(10 per cent)-ligroin, was an amorphous solid, m.p.  $152-162^\circ$ , and Fraction 12, eluted with acetone(15 per cent)-

ligroin, consisted of 2.8 mg. of a gummy solid, m.p. 132–152°; the small quantity of substance in each case did not permit of purification and identification. Efforts to induce crystallization in all of the other eluates proved unavailing.

#### SUMMARY

A total of 250 mg. of purified  $\alpha$ -estradiol was administered intramuscularly to a normal male subject in order to ascertain the nature of the urinary excretory products. Recovered unchanged were 9.8 mg. (3.9 per cent), while oxidized to estrone (isolated as such) were 16.2 mg. (6.4 per cent). No estriol or  $\beta$ -estradiol was obtained.

Thorough exploration of the urine by systematic fractionation and chromatographic analysis failed to separate any other compounds which could be recognized as estrogen metabolites. Isolated were the usual steroids of normal male urine, androsterone, dehydroisoandrosterone, etiocholan-3( $\alpha$ )-ol-17-one, pregnane-3( $\alpha$ ),-20( $\alpha$ )-diol, and cholesterol, together with very small quantities of three unidentified substances.

The fate of the remaining 90 per cent of the hormone, which is inactivated in the body, is discussed.

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# A METHOD FOR THE DETERMINATION OF PHYTATE PHOSPHORUS IN BLOOD

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The recent finding of phytic acid in the erythrocytes of birds and turtles (1) makes desirable a method suitable for the determination of this substance in small amounts of blood. Such measurements must be made in the presence of other organic phosphorus compounds and of variable amounts of inorganic phosphorus present in or likely to be formed in erythrocytes. In searching for such a method we tested a number of procedures based on the precipitation of phytic acid with iron (2-5). None of these procedures was found to be satisfactory, the iron precipitates being contaminated with inorganic and organic phosphates. Various attempts to improve the separation failed. The method of Michel-Durand (6) originally designed for the analysis of relatively large amounts of phytic acid in plant materials proved more satisfactory, and after certain modifications was successfully adapted to the analysis of small samples of blood. In the procedure here described phytate is precipitated from trichloroacetic acid filtrates as a magnesium salt with the inorganic P and some nucleotide P. After hydrolysis to decompose adenosine triphosphate, the phytate is reprecipitated with calcium acetate at pH 5 and the phosphorus in the precipitate is determined. The method is suitable for the determination of amounts exceeding 0.1 mg. of phytate P.

Data on the phytate phosphorus content of the blood cells of several species of birds and turtles (as well as on the absence of phytate in the bloods of other species) determined by this method have been reported previously (7).

## *Reagents—*

1. 20 per cent trichloroacetic acid. Dissolve 200 gm. of tri-



chloroacetic acid, c.p., in water, dilute to 1 liter with water, and filter. Keep in a refrigerator.

2. Magnesia mixture. Dissolve 120 gm. of magnesium nitrate, c.p.  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , and 100 gm. of ammonium chloride, c.p., in water and make to 1 liter.

3. Approximately 8 N ammonium hydroxide solution. Dilute concentrated  $\text{NH}_4\text{OH}$ , sp. gr. 0.90, with an equal volume of water.

4. Calcium acetate, 25 per cent solution. Dissolve 250 gm. of  $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$  in water, make to 1 liter, and filter.

5. 0.5 N hydrochloric acid. Dilute 41.7 cc. of concentrated  $\text{HCl}$ , sp. gr. 1.19, with water and make to 1 liter.

### *Procedure*

*Deproteinization of Blood*—Mix 2 volumes of 20 per cent trichloroacetic acid and 1 volume of blood with shaking; dilute with 2 volumes of water and mix. Filter and measure an amount of filtrate corresponding to 1.5 to 2.0 cc. of blood into a conical, 15 cc., graduated centrifuge tube. If the volume of cells is low, the concentration of phytate in the filtrate may be increased by using cells instead of whole blood. In this case measure accurately an adequate amount of blood into a graduated centrifuge tube; centrifuge and remove most of the plasma without disturbing the cells; and then precipitate with trichloroacetic acid as described above.

*Precipitation of Phytate*—Add to an aliquot of the trichloroacetic acid filtrate a suitable indicator (such as brom-cresol purple) and 1 cc. of the magnesia mixture reagent. Stir and neutralize by adding 8 N  $\text{NH}_4\text{OH}$  drop by drop. After the change of color, add 1 cc. of the ammonium hydroxide solution and mix. Phytic acid if present separates completely as a characteristic flocculent precipitate. The appearance of the magnesium phytate is readily distinguishable from crystalline magnesium ammonium phosphate, and may serve as a qualitative test for this compound if not too large amounts of inorganic P are present. Place the tube in the refrigerator overnight; then centrifuge and remove the supernatant fluid by suction through a capillary tube. Dissolve the precipitate in 5 cc. of 0.5 N  $\text{HCl}$  and heat the solution in boiling water for 10 minutes. Add 5 cc. of 25 per cent calcium acetate solution to the tube, mix, and place the tube in water at about  $80^\circ$  for a

few minutes to coagulate the precipitate. Centrifuge, discard the supernatant fluid, dissolve the precipitate in 2 cc. of  $N$   $HCl$ , and make to a convenient volume with water.

*Digestion of Calcium Phytate*—Ash and determine the P in aliquots of this solution according to the method of Fiske and Subbarow (8). The phytate is very resistant to ashing and care should be taken to make sure the ashing is complete. During the ashing a precipitate of  $CaSO_4$  may appear but this redissolves as the  $H_2SO_4$  becomes more concentrated. As an alternative procedure, especially for small amounts of phytate, the following method of digestion also may be used. To the precipitate of calcium phytate in the graduated 15 cc. centrifuge tube add 1 cc. of 5  $N$   $H_2SO_4$  and 2 drops of concentrated  $HNO_3$ ; then place the tube in a sand bath and heat overnight at about  $130^\circ$ .

*Influence of Other Acid-Soluble Phosphorus Compounds*—In the absence of phytate, inorganic P even in high concentrations (100 mg. per 100 cc.) was not precipitated by calcium acetate. When both inorganic P and phytate were present, in the proportions found in the bloods of birds and turtles, no interference with the determination of phytate P was noted. In a series of experiments (9) in which enzymatic hydrolysis of phytic acid was measured, the experimental conditions were such that the concentration of phytate P was decreasing and that of the inorganic P increasing correspondingly during the period of the experiment. Measurements of inorganic P and of phytate P at various intervals gave concordant results, showing that no appreciable coprecipitation of inorganic P took place under the experimental conditions described. However, larger concentrations of inorganic P did interfere with the determination of phytate P to a varying extent. The largest errors were found when phosphate was added to goose blood in an amount sufficient to raise the concentration of inorganic P in the blood by 20 mg. per 100 cc. Under such conditions errors of 100 per cent were found. Experiments designed to determine the extent of the coprecipitation were carried out with samples of a solution of sodium phytate to which varying amounts of  $KH_2PO_4$  had been added. After the phytate was precipitated with calcium acetate, both inorganic and total P in the precipitate was determined. The data in Table I indicate that under the conditions specified the coprecipitation of inorganic P with

TABLE I

*Recovery of Phytate P from Solutions of Sodium Phytate Containing Various Concentrations of Inorganic P As  $KH_2PO_4$*

The phytate was precipitated with calcium acetate solution. The error of coprecipitation was corrected by determination of inorganic P in the solution of calcium phytate.

Sample		Found		
Inorganic P	Phytate P	Total P	Inorganic P	Recovery of phytate P corrected
mg.	mg.	mg.	mg.	per cent
0.25	0.256	0.262		
0.50	0.511	0.526	0.028	97.4
0.60	0.409	0.427	0.029	97.2
0.70	0.511	0.544	0.043	97.9

TABLE II

*Effect of Adenosine Triphosphate, Diphosphoglycerate, and Trichloroacetic Acid Filtrate of Rabbit Blood on Determination of Phytate P*

Sample			Phytate P found	
Phytate P	Adenosine triphosphate P	Diphosphoglycerate P		
mg.	mg.	mg.	mg.	per cent
0.194	0.65		0.252	130.2
0.194		0.60	0.280	144.7
0.194	0.65	0.60	0.290	149.9
0.194	0.65		0.200	103.3
0.194		0.60	0.196	101.3
0.194	0.65	0.60	0.196	101.3
0.387	0.65	0.60	0.408	105.4
0.387	0.65		0.390	100.8
0.251	Trichloroacetic acid filtrate corresponding to 2 cc. rabbit blood*		0.231	92.1
0.418			0.389	93.0
0.254			0.249	98.0
0.424			0.401	94.8
0.593			0.586	98.8

The first three samples were analyzed by precipitation with magnesia mixture; the others by the method described in the text.

\* Containing 0.64 mg. of acid-soluble P, of which 0.08 mg. was inorganic P, 0.30 mg. diphosphoglycerate P, and 0.12 mg. adenosine triphosphate P.

phytate might lead to errors of 2 to 6 per cent if not corrected. In another experiment an error of 20 per cent was encountered in the analysis of a sample containing 0.25 mg. of phytate P and 0.35 mg. of inorganic P in a volume of 10 cc. The figures in the last column of Table I indicate, however, that the determination of inorganic P in the solution of the calcium phytate precipitate may afford an adequate correction for the coprecipitation of inorganic P.

TABLE III

*Recovery of P from Solutions of Sodium Phytate\* after Precipitation with Magnesia Mixture*

Sample mg.	Found	
	mg.	per cent
0.387	0.407	105.2
0.209	0.200	95.8
0.139	0.139	99.5
0.093	0.092	99.5
0.046	0.046	98.5
0.023	0.023	99.2
0.012	0.012	99.6
0.005	0.005	102.6

\* Sodium phytate was prepared from commercial phytine (Ciba Pharmaceutical Products, New Jersey) essentially according to the procedure described by Posternak (10) for the extracts of grains. Weighed amounts of this salt, dissolved in water and neutralized with HCl to pH 7, were made to suitable volumes.

The influence of large amounts of adenosine triphosphate and diphosphoglycerate is shown in Table II. The data indicate that some coprecipitation of these substances with calcium phytate occurred. It should be noted, however, that considerably larger amounts of adenosine triphosphate and of diphosphoglycerate were present in the solutions analyzed than are apt to be encountered in bloods. Pure solutions of either substance did not give a precipitate with 25 per cent calcium acetate solution at pH 5. In the presence of amounts of trichloroacetic acid filtrate of rabbit blood corresponding to 2 cc. of blood no evidence of coprecipitation was found, and the yields were similar to those obtained from solutions of pure sodium phytate (see Table II).

*Accuracy and Sensitivity of Method*—Duplicate determinations of phytate P in blood samples show good agreement. About 95 per cent recovery was obtained in the determination of 0.20 mg. quantities of phytate P when precipitated as the calcium salt in a volume of 10 cc. The yield dropped to about 88 per cent when 0.10 mg. quantities were determined. The smallest amount that could be detected as the calcium salt was about 0.02 mg. The precipitation as magnesium salt is considerably more sensitive than the precipitation as calcium salt. The data in Table III show that quantities of 5  $\gamma$  are precipitated quantitatively.

#### SUMMARY

The method of Michel-Durand in which phytic acid is precipitated as the calcium salt has been adapted to the determination of phytate phosphorus in trichloroacetic acid filtrates of blood.

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## THE ADRENAL LIPIDS OF FASTED GUINEA PIGS\*

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The importance of the adrenal lipids was recognized as a result of histochemical studies before adequate methods for their chemical determination were developed. Since 1928, however, microchemical methods have been available which make it possible to determine the lipid content of a single adrenal of the larger laboratory animals such as the guinea pig and rabbit.

The French workers, using Grigaut's colorimetric method for the determination of cholesterol, have reported the cholesterol content of guinea pig adrenals after the animals received various treatments. Parhon and coworkers (1-3) have shown that the cholesterol content of the adrenals varies inversely with the water content; thyroidectomy and splenectomy cause no change in the cholesterol values; and thyroxine injections also have no effect on the cholesterol content. Manceau (4) reported a loss in both cholesterol and lecithin in the adrenals after anesthetization of the animals with nitrous oxide or chloroform. Mouriquand and Leulier (5) found a decrease in cholesterol in the adrenals of scorbutic guinea pigs.

Baumann and Holly (6) found that infectious diseases caused a decrease in cholesterol content of the guinea pig adrenal, no significant changes occurring in the phospholipid and neutral fat fractions.

\* The adrenals were supplied by Dr. Raymond Whitehead of the Victoria University of Manchester, England, during his tenure as a Fellow of the Rockefeller Foundation in this department. He hopes to publish the histochemical results obtained on the left adrenals, in due course.

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Materna and Januschke (7) determined the total lipid in the guinea pig adrenal by Soxhlet extraction. They found an average of 14 per cent lipid, fresh weight, in the normal males, and 16 per cent in the normal females. In those animals dying of hunger, the average lipid content showed no change. When the animals were treated with chloroform, the total lipid decreased to an average value of 5.5 per cent.

In none of this work had the determination of the separate components of the lipids of single adrenal glands been reported. One or more component might change without changes occurring in the others, or in the total lipid. This possibility was confirmed in a recent paper by Knouff, Brown, and Schneider (8) on the effect of fatigue on the lipid content of guinea pig adrenals. They reported a decrease in ester cholesterol, but no change in the fatty acid, free cholesterol, and phospholipid fractions.

In most of the stresses applied to experimental animals, fasting is one of the important factors involved. Consequently, in our preliminary investigation of adrenal lipids, a thorough study of the effects of fasting on the adrenal lipids of the guinea pig was made.

#### EXPERIMENTAL

100 male guinea pigs weighing from 350 to 450 gm. were used. The animals were divided randomly into five groups of twenty each. One was used as the control group, and these animals were fed a standard diet on which they consistently gained weight. The second group was completely deprived of food for 3 days; the third group was fasted 7 days; and the fourth group was fasted until the animals appeared to be on the verge of death, the period varying from 10 to 14 days. The fifth group was fasted 3 days and then given the standard diet for 7 days. All the animals were weighed daily for a rough check on their nutritional state. The fasted animals were allowed plenty of tap water. At the end of the experimental period, the animals were killed by a sharp blow on the back of the neck. The adrenals were removed immediately, and the right adrenal used for chemical analysis.

The adrenal was cleaned of all extraneous tissue and then weighed to the nearest mg. The whole gland was ground with sand and extracted with 3:1 alcohol-ether according to the method of Bloor (9). The determinations were made on the whole extract, no

duplicate analyses being possible owing to the small size of the gland. The phospholipid (lecithin) was determined by the method of Bloor (9), and the free cholesterol by the method of Kelsey (10). After saponification of the remaining lipids, the ester cholesterol was determined by the Kelsey method (10). The fatty acids from the cholesterol ester and triglycerides were determined by the oxidative method of Bloor (9).

TABLE I

*Mean Weights of Adrenals and Lipid Fractions for Each Experimental Group; Mean Standard Errors, Experiment Standard Errors, and Significance of Treatment*

Treatment	Adrenal weight (right)	Phospholipid	Free cholesterol	Ester cholesterol	Fatty acid
	mg.	mg.	mg.	mg.	mg.
Control.....	91.0 ±6.39	3.69 ±0.299	0.326 ±0.015	1.352 ±0.044	8.35 ±0.521
Fasted 3 days.....	83.5 ±3.58	3.09 ±0.156	0.398 ±0.013	1.042 ±0.020	7.33 ±0.353
“ 7 “.....	89.8 ±4.81	3.30 ±0.258	0.378 ±0.022	1.069 ±0.028	7.84 ±0.447
“ terminal.....	101.0 ±4.23	4.26 ±0.153	0.272 ±0.017	0.774 ±0.042	6.96 ±0.644
“ 3 days, refed 7 days..	87.8 ±3.53	3.55 ±0.162	0.304 ±0.009	1.151 ±0.027	7.49 ±0.380
Experiment standard error*	4.63	0.215	0.016	0.033	0.481
Significance of treatment (F)†.....	1.96	4.32	10.60	39.62	1.20

\* The significance of the difference between any two treatment means may be judged from the experiment standard error by its ratio to  $\sqrt{2}$  times the experiment standard error.

† Significance values for *F* are as follows: 5 per cent level, 2.46; 1 per cent level, 3.51.

### Data

Table I contains the mean weights of the right adrenal glands and the mean weight of each lipid component of these glands for each group of twenty animals. The standard error of each mean is also given. The statistical analysis<sup>1</sup> of the data as a whole is

<sup>1</sup> The statistical analysis of the data was carried out by Dr. C. P. Winsor, Research Assistant Professor, Statistical Laboratory, Iowa State College, Ames, Iowa.



represented by the standard error of the experiment and the significance of the treatment,  $F$ . The  $F$  test of significance was taken from Snedecor (11).

TABLE II

*Intercorrelations of Adrenal Measurements and Partial Correlations (in Parentheses) Holding Adrenal Weight Constant*

	Phospho- lipid	Free cholesterol	Ester cholesterol	Fatty acid
Adrenal weight.....	+0.849	+0.507	-0.017	+0.489
Phospholipid.....		+0.509 (+0.174)	-0.029 (-0.028)	+0.495 (+0.174)
Free cholesterol.....			+0.038 (+0.054)	+0.472 (+0.298)
Ester ".....				-0.093 (-0.097)
Regression coefficient on adrenal weight.....	+0.0394	+0.00176	-0.000122	+0.05077

TABLE III

*Mean Weights for Adrenal Lipid Fractions with Adrenal Weight Constant*

Treatment	Phospho- lipid	Free choles- terol	Ester choles- terol	Fatty acid
	mg.	mg.	mg.	mg.
Control.....	3.67	0.325	1.352	8.33
Fasted 3 days.....	3.37	0.411	1.041	7.69
" 7 ".....	3.33	0.379	1.069	7.88
" terminal.....	3.85	0.254	0.775	6.44
" 3 days, refed 7 days.....	3.66	0.309	1.151	7.64
Experiment standard error.....	0.11	0.014	0.034	0.42
Mean standard error*.....	0.16	0.020	0.048	0.59
Significance of treatment ( $F$ )†.....	3.55	18.36	37.39	2.76

\* Obtained by multiplying  $\sqrt{2}$  times the experiment standard error. The difference between any two means must be at least 2 times this value to be significant.

† Significance values for  $F$  are as follows: 5 per cent level, 2.46; 1 per cent level, 3.51.

From Table I it can be seen that the adrenal weight and the fatty acid values are not affected by fasting. The phospholipid changes, although significant, do not show a definite trend. The ester cholesterol values decrease regularly with the length of the

fasting period, the decrease being definitely significant. The free cholesterol values show a significant increase after 3 days fasting. However, the decrease from the group fasted 7 days to the terminal fasted group is considerable and sufficient to offset the preliminary rise. The difference between the control and the terminal groups is significant, being  $0.054 \pm 0.023$ .

Table II contains the grouped intercorrelations of the adrenal measurements. The total correlations are all significant with the exception of those involving ester cholesterol, none of which is significant. The figures in parentheses indicate the partial correlations, holding adrenal weight constant, for the entire experiment as obtained by the methods of covariance. It is now evident that none of the partial correlations is significant, indicating that the fractions do not tend to vary together, but rather tend to be functions of the adrenal weight.

Table III indicates the mean weights in mg. for the adrenal lipid fractions with the adrenal weight constant. Since the adrenal weight was shown to be positively correlated with the phospholipid, fatty acid, and free cholesterol values, the precision of the estimates of treatment effects can be improved by the removal of this variable. This was done by reducing all figures to a single adrenal weight according to a linear regression. The calculation eliminates the necessity of assuming a direct proportionality between the lipid values and the adrenal weight, which would be the case were the figures calculated as percentages of the adrenal weight. The statistical method is discussed by Fisher (12) and Snedecor (11).

#### DISCUSSION

Although the weight of the animals decreased considerably on fasting, the weight of the adrenals did not change. This indicates that the glands were protected from emaciation owing to their activity as vital organs.

According to the theory of Selye (13), fasting acts as a stress which causes an increase in the activity of the adrenals and, therefore, an increase in the production of cortical hormone. The slight increase in phospholipid content of the adrenals of the fasted animals may indicate an increase in activity. Bloor (14) showed that increased activity in muscles resulted in an increased phospholipid content of these tissues.

The function of the cholesterol, both free and as the ester, in the adrenal cortex is unknown. It is therefore impossible to explain the marked decrease in cholesterol content which occurs during fasting.

The decrease in total fatty acids which occurred in the adrenals of the fasted guinea pigs could be accounted for only in part by the decrease in cholesterol ester. An actual decrease in triglycerides therefore occurred.

#### SUMMARY

The following changes were noted in the right adrenal of fasted male guinea pigs.

1. There was no change in the weight of the adrenal.
2. The phospholipid, in mg. per gland, showed a slight increase.
3. The free and ester cholesterol decreased, the most marked change occurring in the ester cholesterol fraction.
4. The total fatty acid decrease was significant only to the 5 per cent level.

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# THE FLUOROMETRIC DETERMINATION OF RIBOFLAVIN IN URINE AND OTHER BIOLOGICAL FLUIDS

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The increasing frequency with which riboflavin deficiency is being discovered in man has given an impetus to the study of this substance in biological fluids with a view to providing a quick and accurate diagnostic method. For this purpose chemical methods of determination are distinctly to be preferred to the more laborious and time-consuming biological and microbiological methods. Several procedures have been developed for the estimation of riboflavin in urine, either directly or after some method of concentration. The flavin has been measured quantitatively by its color, by use of a step-photometer (1, 2), by its fluorescence in aqueous solutions (3-5) by converting it into lumiflavin which may be measured colorimetrically in chloroform solutions (6, 7), or by its potential as measured with the polarograph (8). The fluorometric method appealed to us because of its simplicity and sensitivity and because a convenient apparatus for measuring fluorescence photometrically is now available.<sup>1</sup> Although fluorometric methods have been criticized, justly so we believe, by a number of workers in this field, we have been able to modify the procedures used in such a way as to avoid recognized difficulties and to obtain accurate reproducible results.

Our early experiments convinced us that two sources of error inherent in all methods in which fluorescence was measured in aqueous solutions were the turbidity of the solution and the formation of gaseous emulsions. The addition of potassium permanganate followed by hydrogen peroxide, a procedure used to oxidize interfering fluorescent substances, results in the formation of

<sup>1</sup> Pfaltz and Bauer fluorophotometer, models A or B.

minute bubbles of oxygen which tend to remain dispersed in the medium. This gives a whitish tint to the riboflavin fluorescence and interferes with the accuracy of the fluorescence measurements. We have been able to avoid this difficulty entirely by extracting the riboflavin and measuring its fluorescence in a non-aqueous medium.

The separation of the riboflavin from the aqueous medium in which it is usually measured is accomplished by the addition of pyridine and by subsequent saturation of the mixture with anhydrous  $\text{Na}_2\text{SO}_4$ . When appropriate quantities of this salt are added, the pyridine and water are no longer miscible and the pyridine separates out as a surface layer, carrying practically all of the riboflavin with it. By the addition of butyl alcohol, which is miscible with pyridine but not with water, the separation of the riboflavin from the water phase can be made complete. The riboflavin is readily measured in the pyridine-butyl alcohol solution by fluorophotometry.

Our procedure is applicable to either the direct or indirect estimation of riboflavin in urine. The indirect method, involving adsorption and subsequent elution of riboflavin, is usually recommended for the purpose of concentrating the sample when the concentration is less than 0.1  $\gamma$  per cc. or when the urine is deeply pigmented, since a high concentration of urinary pigments interferes appreciably with the passage of light. Under both these conditions our modification is advantageous, since the interfering pigments and the substances responsible for the opacity of urine are highly water-soluble and are not carried over into the butyl alcohol-pyridine phase in significant quantities. We find no difficulty in measuring directly the riboflavin concentration of urines containing as little as 0.05 to 0.1  $\gamma$  per cc., although it is our practice to use the indirect method when the concentration is less than the latter figure. In our indirect method we have followed the procedure of Ellinger and Koschara (9) who adsorbed riboflavin from urine with lead sulfide and eluted it with pyridine and acetic acid. Others have used fullers' earth with success as an adsorbent. Our direct and indirect methods are given in detail below.

*Direct Method for Determination of Riboflavin in Urine*—Urine for riboflavin determinations should be kept in dark bottles to which glacial acetic acid (one-thirtieth of the urine volume) is added

as a preservative. Throughout the manipulations exposure to light should be avoided as far as possible. The quantity of urine taken for analysis should be 5 cc. if a deficiency is suspected; in normal individuals a satisfactory determination can be carried out on 1 to 2 cc. samples, diluted to 5 cc.; after the administration of flavin, as in the case of excretion tests, measurements can readily be made on 0.5 cc. or even smaller quantities, diluted to 5 cc.

The 5 cc. sample of diluted or undiluted urine is put into a 30 cc. separatory funnel.<sup>2</sup> 1 cc. of glacial acetic acid and 2 cc. of purified pyridine are added and the vessel is well shaken. For each cc. of urine used one should then add 1 to 2 drops of 4 per cent  $\text{KMnO}_4$ , depending on the concentration of pigment in the sample. This is left for 1 minute to oxidize interfering substances and the oxidation is then terminated by the addition of sufficient 3 per cent hydrogen peroxide to decolorize the solution, usually a quantity comparable to that of the added permanganate. Anhydrous  $\text{Na}_2\text{SO}_4$  is then added, approximately 1 gm. for each cc. of the sample—a total of 5 gm.<sup>3</sup> 10 cc. of butyl alcohol are then added and the whole is shaken vigorously for 2 minutes; it is then transferred to a centrifuge tube and centrifuged at low speed for 10 to 15 minutes.<sup>4</sup> The clear butyl alcohol-pyridine layer separates on the surface of the watery layer. 12 cc. of this are pipetted out

<sup>2</sup> A special type of separatory funnel, capable of being introduced into a 50 cc. centrifuge tube holder, is best employed. Such vessels, known as reaction vessels, are obtainable from E. Machlett and Son, New York.

<sup>3</sup> When anhydrous sodium sulfate is added gradually to the pyridine-water mixture with shaking, it is usually observed that the pyridine phase separates out on the surface when approximately 3 gm. of sodium sulfate have been added. One should be careful not to stop at this point but should add the additional 2 gm. of  $\text{Na}_2\text{SO}_4$ , even though this causes an emulsion in which the two layers are no longer discernible. The subsequent addition of butyl alcohol with shaking will bring about a complete separation of the two liquid phases, the pyridine-butyl alcohol phase above and the water and undissolved  $\text{Na}_2\text{SO}_4$  below. It may be pointed out that the substitution of an aqueous medium in the measurement of fluorescence possesses the additional advantage that fluorescence is somewhat increased. The following figures for the relative intensity of fluorescence of riboflavin in different media are given by Cohen (3): in aqueous solution 94, in pyridine 112.5, in *n*-butyl alcohol 102.

<sup>4</sup> Centrifugation may be carried out in the special separatory funnel designed for that purpose. However, occasionally the undissolved  $\text{Na}_2\text{SO}_4$  blocks the exit of the separatory funnel, in which case the contents must be transferred to another vessel for centrifugation.

into the cuvette of the fluorophotometer. Fluorescence is measured in a Pfaltz and Bauer fluorophotometer (model A or B), the source of light being a standard mercury vapor bulb (General Electric type H3, 85 watts) shielded by a dark blue filter (Jena, No. BG12) and a yellow filter (Jena, No. GG3). The emitted fluorescence is measured after the interposition of an orange filter (Jena, No. OG1) placed directly in front of the lateral photocell.<sup>5</sup>

Since some fluorescence occurs from Rayleigh scattering of the light rays by the solvent itself, even in the absence of a fluorescent solute, it is necessary to obtain a blank reading on a specimen in which the riboflavin has been destroyed. The fluorescence of this blank is then subtracted from that of the specimen containing riboflavin to give the fluorescence of the riboflavin itself. Our procedure for destroying the riboflavin, which may be carried out either on a duplicate sample or on the original sample after the fluorescence has been read, consists in exposing the specimen to a mercury vapor lamp (or to direct sunlight) for an hour or two, depending on the quantity of riboflavin present. With a little experience one can readily ascertain with the naked eye that the riboflavin has been destroyed, for the fluorescence then no longer has a yellowish hue. We have found this method of destroying riboflavin preferable to the use of reducing agents which convert it to the non-fluorescent leuco form, since the latter may be to some extent reoxidized by atmospheric oxygen. Another advantage of our procedure is that only riboflavin is thereby destroyed, whereas reducing agents decolorize other pigments as well and will hence alter the optical properties of the urine sample.

The concentration of riboflavin is determined by comparing the fluorescence of the unknown sample with that of a riboflavin standard, measured at the same time with an exciting light of identical intensity. Before each measurement the cuvette containing the riboflavin standard is introduced into the instrument and the iris diaphragm is adjusted so that the fluorescence registers some arbitrary figure, such as 10 galvanometric units. The cuvette containing the unknown sample is then substituted for the standard and its fluorescence is read without changing the iris

<sup>5</sup> This optical arrangement was developed by Dr. D. J. Hennessy of the Department of Chemistry, Fordham University. Filters similar to the above are obtainable from the Corning Glass Works, Corning, New York.

diaphragm. Since with a constant exciting light the fluorescence is directly proportional to the concentration of riboflavin, the riboflavin concentration of the unknown can be calculated from a simple proportion between the reading of the standard (minus its blank) and the reading of the unknown (minus its blank). In case the concentration of the unknown is such that it cannot be measured on the galvanometer scale, it is possible that a small alteration in the opening of the iris diaphragm will serve to bring the fluorescence reading upon the scale. In such a case new readings must be made of the riboflavin standard at the new position of the iris and the concentration of the unknown calculated from them by means of simple proportion as before. Samples whose riboflavin concentration differs too widely from that of the standard must be diluted or concentrated. If the concentration is so low that a significant reading cannot be made on the scale without a major alteration of the diaphragm, the concentration procedure described below is indicated with a new urine sample. If the sample is too concentrated, an entirely new determination is not necessary; one may simply dilute the pyridine-butyl alcohol extract with a suitable quantity of a diluting fluid consisting of 20 volumes of butyl alcohol, 4 volumes of pyridine, and 1 volume of glacial acetic acid and measure the fluorescence again.

The standard solutions of riboflavin used for reference are prepared as follows: An aqueous solution of riboflavin containing 10 mg. per liter is prepared fresh every week and is kept in the ice box in the dark. A "daily standard" is prepared from this each day that measurements are to be made as follows: A 1:50 dilution of the "weekly standard" is made with distilled water, and 5 cc. of this (containing 1  $\gamma$  of riboflavin) are treated in exactly the same manner as has been described for the 5 cc. sample of urine or diluted urine. The butyl alcohol-pyridine extract of this known solution containing 1  $\gamma$  is used for comparison with the similar extract made from the unknown sample.

The procedure described differs from that employed by some other workers in the fact that each determination is matched against a riboflavin standard. The reasons for this merit some comment. It is quite possible to dispense with such a standard and to determine the riboflavin concentration from measurements of the exciting light and the fluorescent light or from measurements



of the transmitted light and the fluorescent light. Such procedures have obvious advantages; they avoid the use of a perishable standard and also obviate the need for dilution or concentration of samples of unexpectedly high or low concentration. After some study of the matter we abandoned this approach for a number of reasons. These arise from the fact that it is not practically possible, even with the aid of the diaphragm, to obtain a constant exciting light from day to day. Quite apart from variations in the electrical current to the exciting light, which require expensive apparatus to eliminate, the ultraviolet light bulbs undergo progressive deterioration. When one compensates for these variations by means of the iris diaphragm, certain imperfections in the instrument come into play. A strong light and small diaphragm may give the same recorded transmission intensity as a weaker light with a wider iris opening but the two may produce a different recorded fluorescence. It is obvious that these optical difficulties introduce an error into the determination, a significant one in our experience. Another source of error which cannot be ignored is fatigue of photoelectric cells. When one depends on two photocells, a direct and a lateral one, these may not exhibit fatigue to the same extent. Finally, our observations indicate that fluorescence is not a simple linear function of exciting light or transmitted light, as has been claimed (5), but is a somewhat complicated exponential function. In the range of concentrations in which we are concerned increments of exciting light cause a more than proportional increase in fluorescent light. This adds a complication to the calculations. For these reasons we have preferred to standardize our determinations against a riboflavin solution which is measured each time under identical optical conditions, the only calculation involving a relation which is admittedly a linear one: the relation between fluorescence and concentration with a constant exciting light.

The riboflavin determination can be simplified by assuming a constant blank, as was done by Ferrebee. However, since it is possible that other fluorescent substances may at times appear in the urine which are not destroyed by the measures used to destroy riboflavin, we have retained the use of a blank determination which gives added insurance of the reliability of the measurement.

*Indirect Method for Determination of Riboflavin in Urine*—This

is useful for urines with an extremely low riboflavin content, as well as for highly pigmented urines.

Freshly precipitated lead sulfide is prepared by passing  $\text{H}_2\text{S}$  through 40 cc. of a 5 per cent solution of lead acetate in 0.25 per cent acetic acid. The lead sulfide is washed four times with 300 cc. of distilled water, the precipitate being allowed to settle and the supernatant fluid being decanted. To the moist precipitate from 10 to 30 cc. of urine may be added, the mixture being gently agitated for 10 minutes. It is then filtered with slow suction through a Jena glass filter (Schott and Genossen, No. 3G4). The lead sulfide is washed again with three portions of 30 cc. of distilled water, care being taken to leave enough water just to cover the surface of the precipitate, so that cracks do not form in it. 20 cc. of eluting solution are then added and this is allowed to pass through the lead sulfide layer and filter drop by drop under slow suction.

The eluting solution consists of water 70 cc., pyridine 30 cc., and glacial acetic acid 2 cc. To the eluate are added 3 cc. of glacial acetic acid, followed by 2 cc. of 4 per cent  $\text{KMnO}_4$ . After 1 minute the oxidation is checked and the solution decolorized by the addition of 2 cc. of 3 per cent  $\text{H}_2\text{O}_2$ . The eluate is then made up to 30 cc. in volume with water. An aliquot of this (10 cc.) is then taken and treated with solid anhydrous  $\text{Na}_2\text{SO}_4$  and with butyl alcohol (10 cc.), the remainder of the procedure being identical with the direct method.<sup>6</sup>

*Measurement of Riboflavin in Other Biological Fluids*—The methods described above are readily adapted to the determination of riboflavin in blood serum and in milk. The proteins of blood serum are precipitated by the addition of 10 cc. of 15 per cent trichloroacetic acid to 3 cc. of serum. 5 cc. of the trichloroacetic acid filtrate are then analyzed for riboflavin by the direct procedure described for urine.

The quantity of riboflavin in the blood serum of normal individuals is minute, so minute that only traces can be demonstrated and often none at all. The method is, however, useful in follow-

<sup>6</sup> In calculation of the concentration of riboflavin it should be noted that in the case of the indirect method the butyl alcohol-pyridine sample in which the fluorescence is measured represents only one-third of the riboflavin present in the urine sample.

ing the disappearance curve of riboflavin after an intravenous injection. Our observations with this procedure will be described elsewhere.

TABLE I

*Recovery of Riboflavin from Known Solutions, Treated by Indirect Method of Adsorption on Lead Sulfide, As Read in Pyridine-Water Eluate and in Butyl Alcohol*

Riboflavin added	Recovery in pyridine-water eluate	Recovery after butyl alcohol extraction
$\gamma$	$\gamma$	$\gamma$
2	1.5	2.0
2	1.54	1.95
4	3.3	3.96
4	3.5	3.97
6	5.4	5.89
6	5.46	5.93
8	7.4	7.88
8	7.6	7.98

TABLE II

*Recovery of Riboflavin Added to Urine by Direct and Indirect Method*

Subject	Quantity of urine used	Riboflavin added	Riboflavin recovered	
			Direct method	Indirect method
	cc.	$\gamma$	$\gamma$	$\gamma$
A	5	0		2.7
"	5	4		6.5
"	5	6		8.7
"	5	8		10.8
B	2	0	1.1	
"	2	2	3.1	
"	2	4	5.0	
"	2	6	7.15	

Our procedure may also be used for the determination of riboflavin in milk. The fat is first separated by high speed centrifugation and the protein precipitated with trichloroacetic acid (2 cc. of skim milk + 8 cc. of 10 per cent trichloroacetic acid). 5 cc. of the filtrate are assayed for riboflavin by the direct method.

*Tests of the Method*—The reliability of our butyl alcohol extrac-

tion procedure was tested on known riboflavin solutions of different concentrations. Comparisons were made with the usual

TABLE III

*Comparison of Direct and Indirect Methods for Estimation of Riboflavin in Urine*

Subject	Volume of urine used	Riboflavin concentration	
		Direct method	Indirect method
	cc.	$\gamma$ per cc.	$\gamma$ per cc.
W. F.	1	0.45	0.50
K. Z.	1	0.95	1.0
C. T.	1	3.9	4.0
N. D.	2	4.8	5.0
A. J.	2	1.80	1.70
E. B.	2	2.30	2.35
H. G.	5	0.36	0.35
L. K.	5	0.60	0.62
V. N.	5	0.20	0.21
A	5	2.0*	2.0
		2.0*	2.0
"	10	4.05*	4.0
		3.94*	4.05

\* Duplicates.

TABLE IV

*Comparison of Indirect Fluorometric Method of Riboflavin Estimation with Bacteriological Method of Snell and Strong (10)*

Case No.	Values by indirect method	Values by bacteriological method of Snell and Strong
	$\gamma$ per cc.	$\gamma$ per cc.
1	15.0	15.25
2	3.60	4.41
3	4.20	4.61
4	0.105	0.10
5	1.86	2.65
6	5.70	5.17
7	3.0	2.73
8	4.15	4.19
9	0.04	0.042
10	0.35	0.04

procedure (measurement in pyridine-water) and with subsequent butyl alcohol extraction. The results are shown in Table I. It is

apparent that accurate recovery is not obtained if the riboflavin is measured in pyridine-water eluates.

Our direct and indirect procedures were checked by the addition of riboflavin to urine samples. It may be seen from Table II that added riboflavin may be quantitatively recovered by either method.

Comparative measurements on different urines analyzed by the direct and indirect methods are given in Table III. It may be noted that the direct method is quite as accurate as the indirect method even when the quantity of urine is as large as 10 cc.

In Table IV are shown comparative analyses<sup>7</sup> of the riboflavin concentration of different specimens of urine made by our indirect method and the bacterial growth method of Snell and Strong (10).

Our clinical observations on normal individuals and subjects with riboflavin deficiency will be reported in another publication (11).

#### SUMMARY

An analytical procedure is described for the estimation of riboflavin in biological fluids based on its fluorescence in a non-aqueous medium, butyl alcohol. The advantages of this procedure are discussed.

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<sup>7</sup> The analyses by the bacterial growth method were made by Dr. Isbell and Dr. Wooley of the National Institute of Health, to whom we wish to express our indebtedness.

## CREATINE FORMATION IN THE CHICK

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Earlier reports have shown that glycine is required in the diet for optimum growth of the chick (1, 3, 8). An important function of glycine appeared to be that of a precursor of creatine which was found to be more active than glycine (or acetates) in promoting growth of the chick (1). These results have been confirmed and extended to include the rôle of arginine in creatine formation (7).

Studies on creatine synthesis in the chick were continued with the view of examining also the influence of glycoeyamine and creatinine on the rate of growth of the chick and on the creatine and creatinine content of muscle, liver, and kidney tissue. The results are the subject of the present report.

### *Methods and Results*

The care and housing of chicks and the method of conducting tests have been described (3, 2, 14). The glycine-deficient diet used for the present work contained per 100 gm., casein 20, dried brewers' yeast 10, water-washed and deboned fish meal 10, cod liver oil 1, wheat germ oil 3, Wesson (refined cottonseed) oil 1, gum arabic 7.5, choline chloride 0.2, tricalcium phosphate 2.5, calcium carbonate 1, sodium chloride 1, potassium chloride 0.3, manganese sulfate tetrahydrate 0.1, magnesium sulfate 0.1, sodium silicate 0.1, hexane extract of alfalfa equivalent to 2, and a mixture of equal parts of starch and sucrose to complete the balance. Chicks were reared on this diet from the day of hatching to approximately 10 days of age, then carefully selected for uniform individual and group weight, and continued on the diet for 4 or 5 days more. Groups of ten chicks which showed equivalent rates of gain during

the latter period were then given the dietary supplements. One or two groups were continued on the basal diet without supplement. The gains made by chicks on the supplemented diets over a period of approximately 2 weeks were expressed as per cent of the gains made by chicks on the basal diet only. Except for glycocyamine, the supplements were commercial products. Glycocyamine was synthesized by the method of King (10).

TABLE I  
*Growth-Promoting Effects of Glycine and Other Compounds in  
Glycine-Deficient Chick Ration*

Supplement to basal diet	Level	No. of tests	Basal gain made
	<i>per cent</i>		<i>per cent</i>
None		8	100
Glycine	1.0	4	114
Glycocyamine	1.2	2	114
Creatine hydrate	1.5	7	134
Creatinine	1.5	4	122
Arginine hydrochloride	1.0	1	128
Glycine } Arginine }	1.0 } 1.0 }	4	135
Gelatin	10.0	2	131
Glycine } Guanidine }	1.0 } 1.0 }	2	103
Glycine } Urea }	1.0 } 1.0 }	1	113
Glycolic acid } Urea }	1.0 } 1.0 }	1	104
Choline chloride	0.25	1	101

*Growth-Promoting Effects of Supplements*—The growth-promoting effects of glycine and a number of compounds tested as substitutes for glycine are illustrated by the results given in Table I. These tests have been repeated several times in most cases and have given sufficiently consistent results so that comparisons of the growth-promoting effects seem justified. Glycine, glycocyamine, and creatinine in equivalent quantities induced a definite and approximately equivalent increase in the rate of gain. Creatine was superior to these, however, since less than an equivalent quantity (which would be 2 per cent creatine hydrate) caused a greater

rate of gain. This same comparatively high rate of gain was produced by a combination of glycine and arginine, and by gelatin which is a good source of these amino acids. Extra arginine also improved the rate of growth, although it had not been expected that the basal diet would be lacking in arginine.

Tests were conducted in which the basal diet was fed with and without the choline supplement, and the basal diet plus 1.5 per cent creatine hydrate was also fed with and without the choline supplement. The gains made were entirely unaffected by the

TABLE II

*Effect of Glycine and Other Dietary Supplements on Creatine and Creatinine Contents of Chick Muscle Tissues*

Supplement to basal diet	Level	Creatine*		Creatinine*	
		Leg muscle	Breast muscle	Leg muscle	Breast muscle
	<i>per cent</i>	<i>mg. per gm.</i>	<i>mg. per gm.</i>	<i>mg. per gm.</i>	<i>mg. per gm.</i>
None		3.26	3.48	0.13	0.16
Glycine	1.0	3.51	3.97	0.17	0.21
“	1.0	3.80	4.42	0.20	0.18
Arginine monohydrochloride	1.0				
Gelatin	10.0	4.33	4.53	0.19	0.29
Creatine hydrate	1.5	4.46	4.84	0.25	0.27
Creatinine	1.5	4.87	4.87	0.31	0.47
Practical diet	100.0		4.71		0.15

\* Determined on composite samples from nine or more chicks per group. Not corrected for non-creatine-creatinine chromogens.

presence or absence of the choline supplement, but creatine produced the usual marked increase in the rate of gain. The basal diet evidently contains sufficient choline and the growth increases noted in these experiments are, therefore, not related to any choline-like action (9) on the part of the supplements.

*Effects of Supplements on Muscle Creatine Content*—Composite muscle tissue samples from groups of chicks were analyzed for creatine and creatinine by the modified Jaffe method (13). Typical results may be given in the form of data from one experiment (Table II). These data are relative rather than absolute, since we were not able to determine and correct for the non-creatinine



chromogens at the time. Subsequent analyses have shown, however, that the unknown chromogens in muscle tissue are negligible, amounting to less than 5 per cent of the creatine + creatinine content.

The results (Table II) showed certain differences which were consistent with the view that glycine and arginine are precursors of creatine (5, 6). The muscle creatine content of chicks was slightly increased by the glycine supplement. This increase was greater with the more effective growth-promoting supplements; namely, the glycine-arginine combination, gelatin, and creatine. The creatine values obtained from the groups with the most effective supplements were comparable to those of chicks fed a practical rearing diet and were regarded as "normal." Similar but slight increases occurred in the muscle creatinine content which remained, however, only a small fraction of the creatine content.

An interesting result was the normal creatine content of the groups fed creatinine. Creatinine, however, was consistently less effective than creatine from the standpoint of growth promotion. That poorer growth in the case of creatinine was not due to a toxicity was indicated by the results of a separate experiment in which creatinine levels of 0.75 to 3.0 per cent in the diet gave no evidence of harmful effect and the growth rate increased with the creatinine level. Creatinine feeding also caused an increase in muscle creatinine content, but this increase was very small in comparison to the simultaneous increase in muscle creatine. The creatine content of breast muscle tended to be slightly higher than that of leg muscle, especially in the lower ranges.

In addition to the data in Table II it has been found that the combinations of glycine with guanidine and of glycine with urea caused an increase in muscle creatine equivalent only to that from glycine alone. Glycolic acid plus urea caused no increase.

*Effects of Supplements on Creatine and Creatinine Content of Breast Muscle, Liver, and Kidney*—In the experiments, the specific creatinine and creatine in breast muscle, liver, and kidney tissue were determined according to the bacterial enzyme method (13). Representative results, given in Table III, are in agreement with those of Table II in respect to muscle creatine. Particularly noteworthy is the elevation of muscle creatine by dietary glycocyamine to a value comparable with those obtained with creatine and

creatinine. Even more striking is the comparatively huge (20-fold) increase in liver creatine content produced by feeding glycocyamine.<sup>1</sup>

Creatine feeding slightly raised the creatine content of liver and kidney tissue as well as the muscle creatine content. There was no distinct elevation of tissue creatinine.

TABLE III

*Effect of Glycocyamine, Creatine, Creatinine, and Arginine in Diet on Creatine and Creatinine Contents of Chick Tissues*

Supplement to basal diet	Level	Tissue	Creatine*	Creatinine*
	<i>per cent</i>		<i>mg. per gm.</i>	<i>mg. per gm.</i>
None		Breast muscle	3.74	0.06
		Liver	0.09	0.01
		Kidney	0.05	0.01
Glycocyamine	1.2	Breast muscle	5.00	0.10
		Liver	2.04	0.04
		Kidney	0.31	0.01
Creatine hydrate	1.5	Breast muscle	4.83	0.09
		Liver	0.42	0.01
		Kidney	0.50	0.02
Creatinine	1.2	Breast muscle	5.19	0.16
		Liver	0.12	0.12
		Kidney	0.17	0.21
Arginine monohydrochloride	1.0	Breast muscle	4.04	0.06
		Liver	0.10	0.01
		Kidney	0.12	0.01
Practical diet	100.0	Breast muscle	4.40	0.07
		Liver	0.10	0.04
		Kidney	0.32	0.01

\* Determined on composite samples from a group of ten chicks. Corrected for non-creatine-creatinine chromogens.

Creatinine feeding raised the muscle creatine content to the highest value, as it did in most of the preceding tests, but had no particular effect on liver or kidney creatine. There was a small but distinct rise in creatinine in all tissues.

<sup>1</sup> Under the conditions of the methods of analysis, glycocyamine yielded a color reaction equivalent to 17 per cent of the color reaction of creatine. In both cases the color reaction was destroyed by prior treatment with the bacterial enzyme. It is possible that some of the apparent creatine content of the liver was due to the presence of glycocyamine.

Arginine caused a slight increase in muscle creatine content, which would be expected if its action is that of a precursor limited in effect by the deficiency of another precursor; *i.e.*, glycine.

#### DISCUSSION

The increased rate of growth upon addition of glycine or arginine shows that the basal diet did not contain optimum levels of either of these amino acids. Either glycine or arginine alone caused a slight increase in muscle creatine content. When glycine and arginine were fed together, the growth-promoting effect and the elevation of muscle creatine were most marked. Gelatin, a good source of both of these amino acids, gave similar results. These findings are in harmony with the view that glycine and arginine are specific precursors of creatine (5, 6). It was the demonstrated importance of glycine in creatine formation which first led us to test the latter as a substitute for glycine in chick diets (1).

Unlike the rat, the chick requires dietary arginine even for maintenance (12), and it has a particularly high requirement (4, 12). It also requires dietary glycine for optimum growth (1, 3, 8). The questions of arginine-sparing action and of glycine-sparing action thus become of particular importance for the chick. The marked growth-promoting effect of creatine may be most simply explained on a glycine- and arginine-sparing basis. This explanation has been advanced also by Hegsted *et al.* (7).

Evidence that dietary creatinine was converted to creatine by the chick is found in the resulting high value of muscle creatine as compared to the small value of muscle creatinine. The growth-promoting effect of creatinine thus becomes understandable upon the same glycine- and arginine-sparing basis as that of creatine. The intermediate magnitude of the growth-promoting effect of creatinine may indicate an only moderate rate of conversion of creatinine to creatine, although such conversion appears sufficiently rapid to maintain muscle creatine values at a maximum. The question of creatinine hydrolysis in the rat has been controversial. On the other hand, our results show that in the chick such conversion of creatinine to creatine may take place quite readily.<sup>2</sup>

<sup>2</sup> An alternative explanation for the effect of dietary creatinine is that the presence of such relatively large quantities of creatinine in the organism may greatly reduce the rate of the biological process by which creatine is converted into creatinine.

Glycocyamine, in accordance with the present theory (5, 6) was evidently converted to creatine. This conversion was indicated by muscle creatine values comparable to those obtained from creatine and creatinine feeding. The extremely high liver creatine content resulting from glycocyamine feeding is in agreement with the view that this organ completes the conversion of glycocyamine to creatine (6). It further suggests either a high degree of retention of glycocyamine in the liver or that considerable methylation of glycocyamine continues in the excised liver sample prior to refrigeration and analysis. The growth-promoting effect of glycocyamine was intermediate like that of creatinine, although it might be expected that glycocyamine would have a sparing action on glycine and arginine equivalent to that of creatine.

It is conceivable that the dietary glycocyamine may have placed an excessive demand upon the methylating capacity of the chick, thus disturbing normal metabolism and perhaps consuming methionine to the detriment of other growth processes. However, when extra methionine was fed with glycocyamine in a separate experiment, the growth rate was not noticeably improved.

It has been shown that creatine does not substitute for methionine in chick diets that are deficient in methionine to the extent that growth is prevented (11). In the present experiment the dietary additions of glycine plus arginine or of gelatin (which contains no methionine) resulted in as good gains as with creatine, and approximately equal muscle creatine values. The growth-promoting effect of creatine, therefore, would not appear to involve a sparing action on methionine.

That the possible participation of methionine in creatine synthesis in the chick may not be readily demonstrated by the methods employed in this study is shown by the results of experiments with a semipurified diet (to be described later) in which the only sources of protein were arachin and gelatin. Without an addition of methionine, chicks failed to grow on this diet but the muscle creatine content was 5.3 mg. per gm. The addition of methionine to the diet resulted in good growth; the muscle creatine, however, was 4.6 mg. per gm. A deficiency of methionine serious enough to prevent growth did not diminish the muscle creatine content.

Among the unsupplemented groups, chicks have developed a condition of general weakness and muscular attenuation leading in extreme cases to prostration. On the other hand, the most ef-

fectively supplemented groups have remained vigorous and active. This apparent muscular dystrophy of the chick which we have previously reported (1) and which has been confirmed (7) is accompanied by a subnormal muscle creatine content evidently related to the deficiency of creatine precursors in the basal diet. In this condition liver creatine remains approximately normal but kidney creatine appears to be lowered as well as muscle creatine.

The writers are grateful for opportunities to discuss this work with Professors C. L. A. Schmidt, R. Schoenheimer, H. Borsook, and H. H. Beard, and for preparations of the NC creatinine organism kindly provided by Professors Borsook and Beard.

#### SUMMARY

1. Glycine, arginine, glycine plus arginine, gelatin, glyco-cyammine, creatine, and creatinine in the diet of the chick lead to increased muscle creatine content and rate of growth.

2. The results are in harmony with the view that glycine and arginine are specific biological precursors of creatine.

3. In a deficiency of these precursors, the muscle creatine content of the chick becomes abnormally low and a condition of muscular attenuation and profound weakness develops.

4. Severe methionine deficiency does not reduce the muscle creatine content below normal.

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# THE PARTIAL REPLACEMENT OF DIETARY METHIONINE BY CYSTINE FOR PURPOSES OF GROWTH\*

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In an earlier paper Womack, Kemmerer, and Rose (1937) presented convincing evidence for the dietary indispensability of methionine. Cystine, on the other hand, was shown to be a dispensable component of the food.

These observations appeared to be in conflict with the well known fact that the addition of cystine to certain low protein diets is followed by an increase in the rate at which the animals gain. In order to account for this apparent discrepancy the suggestion was made that cystine may be able "to function in place of part of the methionine, but not as a substitute for all." In other words, it was assumed (1) that both methionine and cystine are necessary *tissue* components, (2) that the methionine is derived only from the food, and (3) that the cystine may either be of exogenous origin or may be synthesized from methionine *in vivo*. According to this concept, the addition of cystine to a diet containing an insufficient amount of methionine alone, or insufficient amounts of both cystine and methionine, would be expected to improve the quality of the food by sparing the conversion of methionine into cystine. The experiments outlined below were designed to test this hypothesis.

## EXPERIMENTAL

Two amino acid mixtures were employed; namely, Mixture XV-a and Mixture XX-a. The former, which was used in the first of the present series of experiments, has already been de-

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TABLE I  
*Composition of Diets, Per Cent*

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Amino acid Mixture XV-a*..	27.5	26.6	26.7			
“ “ “ XX-a†..				19.7	19.7	19.7
Threonine ( <i>d</i> (-)-).....	0.7	0.7	0.7			
“ ( <i>dl</i> -).....				1.4	1.4	1.4
Dextrin.....	19.5	19.3	19.8	26.3	26.1	25.9
Sucrose.....	15.0	15.0	15.0	15.0	15.0	15.0
Salt mixture‡.....	4.0	4.0	4.0	4.0	4.0	4.0
Agar.....	2.0	2.0	2.0	2.0	2.0	2.0
Cod liver oil.....	5.0	5.0	5.0	5.0	5.0	5.0
Lard.....	26.0	26.0	26.0	26.0	26.0	26.0
Cystine ( <i>l</i> (-)-).....			0.5	0.4	0.4	0.4
Methionine ( <i>dl</i> -).....	0.3	1.4	0.3	0.2	0.4	0.6
	100.0	100.0	100.0	100.0	100.0	100.0

\* Womack, Kemmerer, and Rose (1937).

† Rose and Rice (1939).

‡ Osborne and Mendel (1919).

TABLE II

*Total Changes in Weight and Total Food Intakes of Experimental Animals*

The vitamin B factors were furnished in the form of two pills daily, each containing 75 mg. of a commercial cereal concentrate (vitamin B complex liquid type 2, Vitab Products, Inc., New York).

Rat No. and sex	Days	Total gain in weight	Total food intake	Diet No.	Supplement
		<i>gm.</i>	<i>gm.</i>		
2650 ♀	12	3	48	1	0.3% <i>dl</i> -methionine
	32	49	195*	2	1.4% “
2651 ♂	12	1	44	1	0.3% “
	32	43	145	2	1.4% “
2652 ♀	12	4	58	1	0.3% “
	32	20	142	3	0.3% “ + 0.5% <i>l</i> -cystine
2653 ♂	12	4	74*	1	0.3% “
	32	25	176*	3	0.3% “ + 0.5% <i>l</i> -cystine
2654 ♂	12	3	48	1	0.3% “
	32	21	140	3	0.3% “ + 0.5% <i>l</i> -cystine
2655 ♂	12	1	48	1	0.3% “
	32	19	140	3	0.3% “ + 0.5% <i>l</i> -cystine
2656 ♀	44	10	191	1	0.3% “
2657 ♂	44	9	162	1	0.3% “

\* Some food scattered.

scribed by Womack, Kemmerer, and Rose (1937). It was devoid of cystine, methionine, and threonine. These amino acids were added separately to the diets in the desired amounts, as shown in Table I. The food was furnished *ad libitum*.

The results of the first tests are shown in Chart I and Table II. For 12 days each of the animals received a diet (Diet 1) containing 0.3 per cent of *dl*-methionine and no cystine. This permitted small increases in weight (1 to 6 gm.). At the end of this period, two of the subjects (Rats 2656 and 2657) were continued upon this ration, two (Rats 2650 and 2651) were transferred to a régime containing 1.4 per cent of *dl*-methionine without cystine (Diet 2), and four (Rats 2652 to 2655) were given a diet carrying 0.3 per cent of *dl*-methionine and 0.5 per cent of *l*-cystine (Diet 3). As may be seen from Chart I and Table II, the rats on the food containing 0.3 per cent of *dl*-methionine continued to grow very slowly (9 and 10 gm. in 44 days). On the other hand, the addition of 0.5 per cent of *l*-cystine induced gains of 19 to 25 gm. in 32 days. The higher level of methionine caused even better gains (43 and 49 gm. in 32 days). The former investigation of Womack, Kemmerer, and Rose (1937) demonstrated that "cystine is incapable of promoting growth when methionine is absent from the food," and that "Its presence does not improve the quality of a ration carrying an adequate amount of methionine." These facts, taken in conjunction with the present observations, render it apparent that *cystine stimulates growth only when methionine is furnished in suboptimal amounts*.

That the increases in weight are more closely related to the methionine than to the cystine content of the food is shown by the results of the second series of experiments. Here amino acid Mixture XX-a was used. This was identical in composition with that described by Rose and Rice (1939) except that the threonine was added directly to the diets instead of being incorporated in the amino acid mixture. The cystine content of each ration (Diets 4 to 6, Table I) was maintained at 0.4 per cent, which is undoubtedly a liberal quantity of this amino acid, and the methionine was introduced at levels of 0.2, 0.4, and 0.6 per cent respectively. The diets were furnished *ad libitum*.

A litter of nine animals was divided equally among the three diets, and maintained thereon for a period of 28 days. The results are summarized in Table III. As will be seen, each increase in

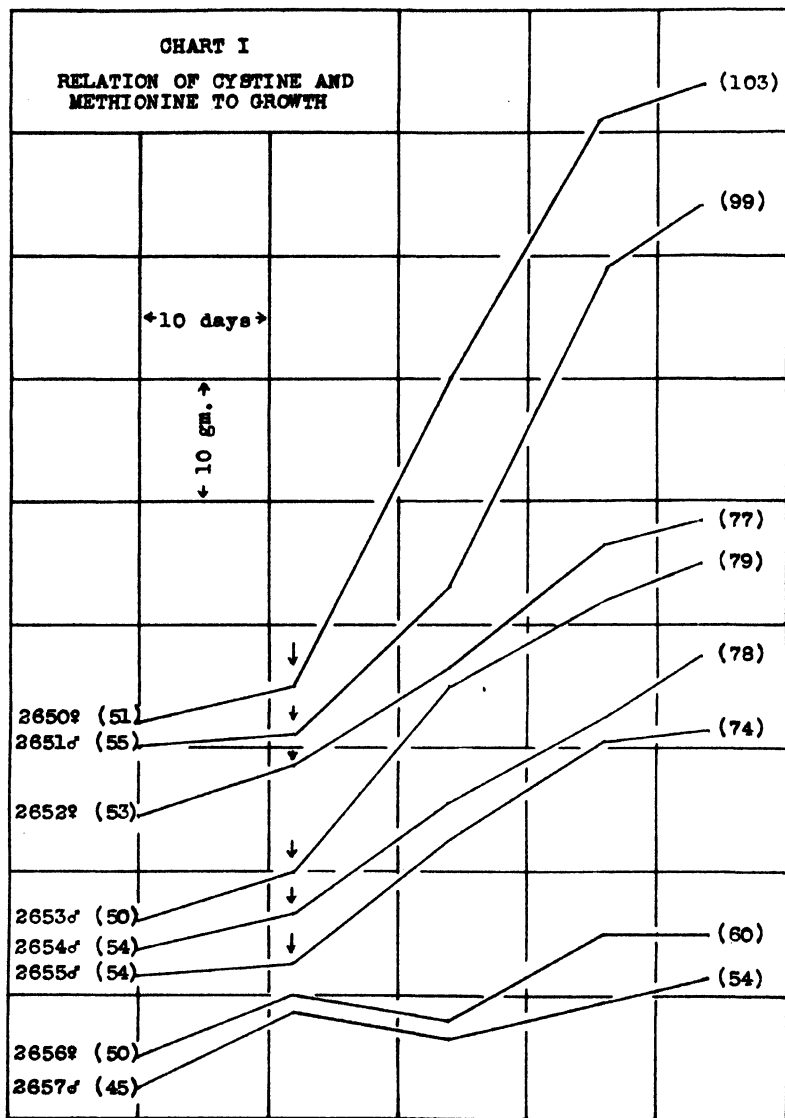


CHART I. The numbers in parentheses denote the initial and final weights of the rats. At the beginning of the tests the animals were placed upon a diet containing 0.3 per cent of *dl*-methionine and no cystine (Diet 1). The arrows indicate the points at which dietary changes were made as follows: Rats 2650 and 2651, 1.4 per cent of *dl*-methionine (Diet 2); Rats 2652 to 2655 inclusive, 0.3 per cent of *dl*-methionine plus 0.5 per cent of *l*-cystine (Diet 3). Rats 2656 and 2657 were maintained on Diet 1 throughout.

methionine led to an augmentation in the growth rate. Other experiments not included here indicate that growth acceleration occurs with each increment in methionine until the amount of this amino acid reaches 0.5 per cent of the ration. Further additions are without effect. When the diet is devoid of cystine, the minimal amount of methionine necessary to induce optimum growth is 0.6 per cent (Rose and Womack, unpublished data). Evidently,

TABLE III

*Total Changes in Weight and Total Food Intakes of Experimental Animals*

The experiments covered 28 days. The vitamin B factors were furnished in the form of two pills daily, each containing 50 mg. of tikitiki and 75 mg. of milk concentrate.

Rat No. and sex	Total gain in weight	Total food intake	Diet No.	dl-Methionine supplement (+ 0.4% l-cystine)
	gm.	gm.		per cent
3643 ♂	23	111	4	0.2
3644 ♂	19	99	4	0.2
3645 ♀	16	90	4	0.2
3646 ♂	35	117	5	0.4
3647 ♀	43	149	5	0.4
3648 ♀	35	117	5	0.4
3649 ♂	49	121	6	0.6
3650 ♀	51	149	6	0.6
3651 ♀	51	132	6	0.6

therefore, only a small portion (approximately one-sixth) of the methionine requirement can be satisfied by cystine.

## SUMMARY

The results of experiments involving the use of otherwise adequate diets containing varying proportions of cystine and methionine demonstrate that cystine is capable of stimulating growth only when methionine is present in suboptimal amounts.

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## THE SYNTHESIS OF CYSTINE IN VIVO\*

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The fact that animals grow at maximum rates upon diets devoid of cystine (Womack, Kemmerer, and Rose, 1937) appears to warrant the inference that this amino acid can be synthesized by the tissues. Furthermore, a large body of evidence is available favoring the presumption that the precursor of cystine is methionine. Thus, Brand, Cahill, and Harris (1935) and Lewis, Brown, and White (1936) report that the administration of methionine to cystinuric patients is followed by the excretion of extra cystine in the urine. Cystine, administered under like circumstances, is almost completely oxidized, and hence does not contribute to the cystine output. Moreover, methionine is said to be an effective agent in promoting the detoxication of bromobenzene (White and Jackson, 1935; Stekol, 1937, *a*), naphthalene (Stekol, 1937, *b*), iodoacetic acid (Simon and White, 1938), and methyleholanthrene (White and White, 1939). Heard and Lewis (1938) found that the administration of methionine to rats upon a ration low in the sulfur-containing amino acids is accompanied by a slightly increased production of hair, containing a higher percentage of cystine than is found in the hair of animals receiving the basal diet alone. More recently, Brown and Lewis (1941) have observed increases in the cystine content of blood plasma ultrafiltrates following the oral or subcutaneous administration of methionine to normal rabbits.

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† The experimental data in this paper are taken from a thesis submitted by Thomas R. Wood in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

Unfortunately, several of the above experiments involved the use of diets containing appreciable amounts of cystine. In so far as this was true, the data are open to serious criticism. It is hazardous to postulate the synthesis of an amino acid as the result of feeding trials in which the compound under investigation is not excluded from the food, unless it can be shown that the amount utilized for the purpose in question exceeds the quantity which could have been derived from the diet. This fact is appreciated by Heard and Lewis (1938), who point out that the relation of methionine to keratinization (cystinization) of hair can be demonstrated conclusively only by the use of a ration the amino acid content of which is known exactly.

Because of these considerations, an investigation was undertaken in which the nitrogenous components of the diets were supplied in the form of mixtures of highly purified amino acids. Under these circumstances, the only nitrogen and sulfur of unknown nature present in the food were the small amounts furnished by the concentrates used as sources of the vitamin B factors. As will be shown later, these quantities could not have influenced the outcome of the investigation.

After the present study had been completed, Dawbarn (1938) published the results of experiments demonstrating that in rats receiving a low cystine ration the deposition of the amino acid in the fleece alone was greater than could be accounted for by the cystine intake. This constitutes the first *direct* proof of cystine formation in the animal organism. Shortly thereafter, Beach and White (1939) reached a similar conclusion as the result of analyses of the entire carcasses of rats which had been permitted to grow on a diet low in cystine. With a single exception, each animal was found to have stored in the tissues an average of more than 2 mg. of cystine daily in excess of the amount ingested. Both Dawbarn and Beach and White interpret their data as indicating the transformation of methionine into cystine.

More recently, Tarver and Schmidt (1939) investigated the fate in rats of methionine containing radioactive sulfur, and were able to demonstrate the presence of the isotope in the cystine isolated from the tissues. This provides convincing proof that methionine is the precursor of cystine, at least in so far as the sulfur in the molecule is concerned.

None of the experiments described above furnishes wholly satisfactory information concerning the *extent* of cystine formation *in vivo*. Since our investigation involved the use of a diet more nearly devoid of cystine than any previously employed, the data represent a closer approach to a *quantitative* measure of the synthesis of this amino acid during growth than any previously recorded. From this point of view particularly are the findings of interest.

#### EXPERIMENTAL

The procedure involved the principle made use of by Scull and Rose (1930) in demonstrating the *in vivo* synthesis of arginine. For this purpose, a mixture of amino acids which was devoid of both cystine and methionine was incorporated in two diets, one of which (Diet 1) was supplemented with each of the sulfur-containing amino acids, and the other (Diet 2) with methionine alone. Both diets were administered *ad libitum*. At the beginning of the tests, litter mates of the rats employed in the growth studies were killed and subjected to analysis *in toto* for cystine. The other members of each litter were killed and analyzed after they had received the experimental diets for periods of 36 or 48 days. Obviously, in the animals upon Diet 2 the increments in tissue cystine incidental to growth, after being corrected for the possible traces of cystine present in the vitamin supplements, represent the amounts of the amino acid which were manufactured *in vivo*.

*Composition of Diets*—The composition of the amino acid mixture (Mixture XXI-a) is presented in Table I. Each of the components was shown to be analytically pure, and to be devoid of cystine, as measured by the Sullivan test.

The make-up of the diets is shown in Table II. Their sulfur content was identical, though derived from different proportions of the sulfur-containing amino acids. Furthermore, each diet contained 16.0 per cent of amino acid Mixture XXI-a. This represents the sum of the quantities listed in the third column of Table I. Thus, except for cystine and methionine, the per cent of each physiologically effective amino acid in the food is represented by the corresponding figure in the second column of Table I. Diet 1 contained 11.9 and Diet 2, 12.0 per cent of effective amino acids.



The vitamin B factors were furnished to each animal in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitrogen from these sources amounted to slightly less than 4 mg. We have used these

TABLE I  
*Composition of Amino Acid Mixture*

	Mixture XXI-a	
	Physiologically active amino acids	As used
	gm.	gm.
Glycine.....	0.1	0.1
Alanine.....	0.2	0.4*
Serine.....	0.1	0.2*
Valine.....	1.0	2.0*
Leucine.....	1.2	2.4*
Isoleucine.....	0.8	1.6*
Cystine.....		
Methionine.....		
Proline.....	0.2	0.2
Hydroxyproline.....	0.1	0.1
Aspartic acid.....	0.2	0.2
Glutamic ".....	2.0	2.0
Phenylalanine.....	1.2	1.2*
Tyrosine.....	0.6	0.6
Arginine.....	0.4	
" monohydrochloride.....		0.5
Histidine.....	0.5	
" monohydrochloride monohydrate.....		0.7
Lysine.....	1.2	
" monohydrochloride.....		1.5
Tryptophane.....	0.4	0.4
Threonine.....	0.7	0.7
Sodium bicarbonate.....		1.2
	10.9	16.0

\* Racemic acids.

materials in several hundred tests, and have found them satisfactory for feeding trials of relatively short duration (50 to 60 days). In the present investigation it was essential to know the extent to which they might contribute cystine to the food. Since

preliminary experiments had indicated that they could not be analyzed for cystine without considerable error, we resorted instead to a measure of the organically bound sulfur (total sulfur minus inorganic sulfur). The results showed that the daily intake of organic sulfur from these sources amounted to 0.2 mg. If this were solely in the form of cystine, which undoubtedly was not the case, it would be equivalent to 0.75 mg. of cystine daily from the vitamin pills. Therefore, this figure was used in correcting the increments in tissue cystine, as will be pointed out later.

TABLE II  
*Composition of Diets*

The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract.

	Diet 1	Diet 2
	gm.	gm.
Amino acid Mixture XXI-a	16.0	16.0
Cystine ( <i>l</i> (-)-)	0.2	0.0
Methionine ( <i>dl</i> -)	0.8	1.1
Dextrin	31.0	30.9
Sucrose	15.0	15.0
Salt mixture*	4.0	4.0
Agar	2.0	2.0
Lard	26.0	26.0
Cod liver oil	5.0	5.0
	100.0	100.0

\* Osborne and Mendel (1919).

Four litters of young rats were employed. With Litter 1 the feeding trials were terminated after 36 days, and with Litters 2, 3, and 4 after 48 days. Two or three animals from each litter were sacrificed at the beginning of the feeding trials and analyzed for cystine. The remaining animals in each litter were divided into two groups, one of which received Diet 1 and the other Diet 2. At the end of the feeding periods each animal was weighed, chloroformed, and analyzed for cystine. For this purpose, the gastrointestinal tract was removed and washed out under the tap. The entire carcass was then weighed and ground thoroughly in a meat chopper. The resulting material was placed in a 1000 cc. round

bottom flask, covered with about 700 cc. of acetone, and mixed. After standing for 24 hours, the acetone-water was removed by filtration. This extraction was repeated four times with acetone and four times with ether. The solid material was then removed by filtration and dried in a vacuum oven at 60°. The dry tissue so obtained consisted of a light gray powder which amounted to about 20 per cent of the live weight of the animal.

In the estimation of cystine the tissue powder was hydrolyzed by boiling for 20 hours on a sand bath with 8 times its weight of 40 per cent sulfuric acid. After cooling, the hydrolysate was filtered, and diluted until each cc. contained approximately 1 to 1.5 mg. of cystine. The remainder of the procedure was carried out essentially as described by Rossouw and Wilken-Jorden (1935), the color intensity being measured in a photoelectric colorimeter. Preliminary tests showed that no cystine-containing material was removed by the treatment with acetone and ether, and that additions of cystine to a tissue preparation preceding hydrolysis were recovered quantitatively.

In order to conserve space, the results of one typical experiment only are presented in detail. These are found in Table III. The cystine percentages were calculated on the basis of the tissue weights; *i.e.*, the weights of the carcasses after the alimentary residues had been removed. Rats 3600 and 3601 were the controls which were analyzed at the beginning of the experiment. The other members of the litter received Diet 1 or Diet 2 for 48 days. The average weight of the alimentary contents of the controls was assumed to represent the initial weight of the alimentary residues of each of their litter mates. In like manner, the average percentage of cystine in the controls was used in calculating the initial cystine content of the rats which received the diets.

The *percentage* of cystine in the animals at the expiration of the growth periods was invariably larger than in the controls at the beginning of the experiments. This was found to be true also of arginine (Scull and Rose, 1930), and probably is attributable to a larger proportion of water and skeletal structures in younger than in older rats.

The figures in the last column of Table III, representing the increments in cystine, demonstrate that the subjects increased

their content of this amino acid by values ranging from 229 to 269 mg. No significant difference is to be observed between the animals which received both cystine and methionine (Diet 1) and those which received methionine alone (Diet 2). Nor was there a significant difference in the rate at which the members of the two groups gained in body weight. Of the animals in the four litters, eleven received both cystine and methionine, and showed an average cystine increment of 3.5 mg. per gm. of gain in body

TABLE III

*Increment in Tissue Cystine during Growth, Litter 2*

The feeding experiments were continued for 48 days.

Rat No. and sex	Dietary supplement	Body weight				Cystine content				Cystine incre- ment
		Initial		Final						
		Live	Tissue*	Live	Tissue*	Initial	Final	Initial	Final	
		gm.	gm.	gm.	gm.	mg. per cent	mg. per cent	mg.	mg.	mg.
3600 ♂		56	47.5			259		123		
3601 ♂		58	51.2			231		118		
3592 ♂	Cystine + methio- nine	51	43.4	133	126	245	298	106	375	269
3593 ♂		53	45.4	134	125	245	286	111	357	246
3594 ♀		55	47.4	129	119	245	290	116	345	229
3595 ♂	Methionine	52	44.4	133	121	245	288	109	348	239
3597 ♀		54	46.4	132	124	245	294	114	365	251
3598 ♀		54	46.4	127	117	245	307	114	359	245

\* Weight after removal of the alimentary contents.

weight. The corresponding figure for the fifteen rats which received methionine alone was 3.6 mg.

In Table IV are summarized the data on cystine synthesis for each of the four litters. The values given in the fifth column represent the differences between the increments in tissue cystine and the maximum possible cystine intakes of the subjects on Diet 2. As will be observed, the amount of cystine manufactured by the organism varied from 144 mg. in the 36 day experiment to 307 mg. in one of the 48 day experiments. On a daily basis the cystine synthesis varied from 4 to 6.4 mg. These quantities, though

smaller than the figure representing the average daily synthesis of arginine in the rat (*cf.* Scull and Rose (1930)), are much larger than any hitherto reported for cystine. Obviously, they represent minimal values. On the other hand, they would be increased by only 0.75 mg. each even if it were assumed that none of the organically bound sulfur in the vitamin pills was present as cystine. One may assert, therefore, with considerable confidence that the cystine requirement of the young rat is not far from 4 to 6 mg. per day, depending upon the rate of gain, and that this quantity may be obtained through synthesis alone when the ration is adequate in other respects.

TABLE IV  
*Cystine Synthesis during Growth*

Litter No.	Duration of experiment	Average increment in tissue cystine	Maximum cystine intake*	Average cystine synthesis	Rate of cystine synthesis
	<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg. per day</i>
1	36	171	27	144	4.0
2	48	245	36	209	4.4
3	48	343	36	307	6.4
4	48	324	36	288	6.0

\* Based on the improbable assumption that all of the organic sulfur in the vitamin pills was present as cystine.

Inasmuch as methionine was the only organic sulfur compound available in sufficient proportions to account for the cystine deposition in the tissues of the animals on Diet 2, the conclusion is warranted that the *sulfur* of methionine was utilized in the formation of cystine. It should be borne in mind, however, that neither our observations, nor those of others, indicate necessarily that the carbon chain of cystine has its origin in the carbon chain of methionine. This problem, as well as the mechanism involved in cystine synthesis, still awaits elucidation.

#### SUMMARY

By measuring the increments in tissue cystine of young rats receiving diets containing mixtures of highly purified amino acids in place of proteins it has been shown that 4 to 6 mg. of cystine may be synthesized daily for purposes of growth. No significant

differences are observable in the cystine increments in the tissues nor in the rates of gain in body weight of subjects receiving (1) methionine alone, and (2) methionine plus cystine. Thus, the quantities indicated above appear to represent a close approximation to the cystine requirement of the young growing rat for new tissue formation.

Since in certain of the experiments methionine was the only organic sulfur compound available in sufficient proportions to account for the cystine deposition in the tissues, one is warranted in concluding that the *sulfur* of methionine is utilized in the manufacture of cystine. Neither our observations, nor the published findings of others, indicate necessarily that the carbon chain of cystine originates in the carbon chain of methionine.

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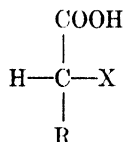
# HOMOLOGOUS SERIES OF $\alpha$ -SUBSTITUTED ALIPHATIC ACIDS

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It is now recognized that every group attached to the asymmetric carbon atom contributes to the total rotation of the molecule. Thus, given an acid of the general type



in which X is a functional group and R is an alkyl group, its rotation will vary not only with variations in X but also with variations in R. The effects of changes in R are due not only to variations in the numerical values of its own contributions but also to variations in its inducing (vicinal) effect on COOH and on X, by virtue of which the contributions of each of these two groups likewise vary. It was observed first by Tschugaëff that the rotations of members of homologous series of substances increase in value, reaching a certain maximum. In our laboratory observations on several groups of homologous substances caused us to modify the conclusion of Tschugaëff to state that the molecular rotations of successive members of configurationally related homologous series progressively move in the same direction. For example, if the first member is levorotatory and the second and third dextrorotatory, the rotation of the first is more levo, and that of the third more dextro, than that of the second (1). It is desirable to extend the observations to a variety of homologous

\* Died September 6, 1940.



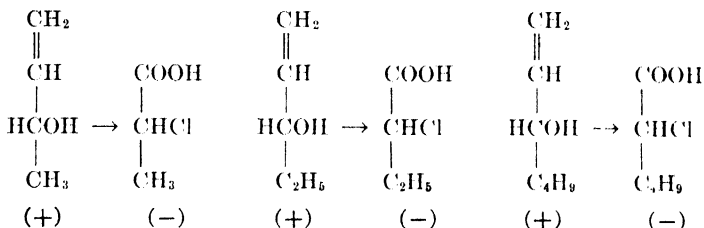
series in order to test whether this property may be accepted as a general property of all homologous series.

The present communication relates to the grouping of  $\alpha$ -bromo aliphatic acids and of  $\alpha$ -amino aliphatic acids into homologous series and the establishment of the maximum rotations of the homologous series of  $\alpha$ -hydroxy,  $\alpha$ -bromo, and of  $\alpha$ -amino acids. Three members of each series were studied; namely,  $\alpha$ -substituted propionic, butyric, and caproic acids.

*Grouping in Homologous Series*—The  $\alpha$ -hydroxy acids were grouped into a homologous series by Levene and Haller (2), who found that homologous  $\alpha$ -hydroxy acids rotate in the same direction.

The  $l(+)$ - $\alpha$ -hydroxy aliphatic acids are configurationally related to the  $l$ - $\alpha$ -amino acids, and thus the homologous series of  $\alpha$ -amino aliphatic acids has the same sign of rotation.

The  $\alpha$ -bromo aliphatic acids were grouped into a homologous series by identical treatment of the  $\alpha$ -amino acids with nitrosyl bromide. It was found that the individual members of this homologous series of  $\alpha$ -bromo aliphatic acids have the same sign of rotation. It may also be expected that homologous series of  $\alpha$ -hydroxy acids should by an identical mode of treatment of its individual members lead to a homologous series of  $\alpha$ -halogeno acids. On the basis of this consideration, it follows from the work of Levene, Mori, and Mikeska (3) that the members of the homologous series of  $\alpha$ -halogeno acids rotate in the same direction. Levene and Haller (4) later confirmed this conclusion on the basis of a different set of experiments.



The members of the homologous series of vinylcarbinols led to a series of  $\alpha$ -chloro acids rotating in the same direction. The identity of the configurations of the halogeno acids can further be substantiated by the fact that, in all acids rotating in the same

direction, induction of the ionized state causes the rotation to shift in the same direction.

In all of the  $\alpha$ -substituted acids, as given in Table I, the sense of the shift in the direction of rotation on passing from the undissociated state of the carboxyl to the ionized state remains the same for each of the individual members of the homologous series (5).

*Resolution of  $\alpha$ -Hydroxy Acids*—Because of the differences in solubility of the individual members of this series and because of the lactide formation in the process of liberation of the free acids, it was found preferable to refer to the rotation of the barium salts in aqueous solution.

Lactic acid has been resolved by Purdie and Walker (6). The maximum rotation of the sodium salt of the  $l(+)$  acid is  $[M]_D^{25} = -11.9^\circ$ . The acid has  $[M]_D^{15} = +3.4^\circ$  ( $c = 10.5$ ).

*$\alpha$ -Hydroxy- $n$ -butyric acid* has been resolved by Guye and Jordan (7) through the brucine salt and by Levene and Haller (4) through the morphine salt. In the present study, the recrystallization of the morphine salt was continued until the solid phase and the filtrate yielded a barium salt of the  $d(-)$ - $\alpha$ -hydroxybutyric acid with a constant rotation of  $[M]_D^{25} = +14.9^\circ$ , somewhat higher than that ( $+13.2^\circ$ ) previously recorded (4).

*$\alpha$ -Hydroxycaproic acid* has been resolved by Levene, Mori, and Mikeska (3) through the cinchonidine salt. The same procedure was followed, and the recrystallization was continued until the rotations of the barium salts obtained respectively from the solid phase and from the mother liquor were practically identical. The rotation of the barium salt of the  $d(-)$  acid was  $[M]_D^{25} = +22.2^\circ$ , which is practically the same as that previously reported.

*Resolution of  $\alpha$ -Bromo Acids*—The resolution of the  $\alpha$ -bromopropionic acid to maximum was accomplished by Ramberg (8) through the cinchonine salt; in homogeneous state it had  $[M]_D^{25} = -43.6^\circ$ .

*$\alpha$ -Bromobutyric acid* was resolved through the brucine salt. The maximum rotation was found to be  $[M]_D^{25} = +59.6^\circ$  (homogeneous). It is peculiar that some time ago Ahlberg (9) failed to resolve the substance through the brucine salt but was successful in accomplishing the task through the strychnine salt, although the maximum rotation obtained by this author was only  $[M]_D^{25} =$

TABLE I  
Maximum Rotations of Acids of the Type  $RCHXCOOH$  ( $X = OH, Br, \text{ and } NH_2$ ) and Derivatives

$\alpha$ -Hydroxy acids	$[M]_D^{25}$ degrees	$\alpha$ -Bromo acids	$[M]_D^{25}$ degrees	$\alpha$ -Amino acids	$[M]_D^{25}$ degrees
<i>l</i> -Lactic acid		$\alpha$ -Bromopropionic acid		<i>l</i> -Alanine	
Na salt in $H_2O$	-11.9	In $H_2O$	+45	In 20% HCl	+13.6
Acid in $H_2O$	+3.4	Na salt in $H_2O$	-11	" $H_2O$	+4.0
		In 30% alcohol	+46		
		" ether	+57		
		Homogeneous	+44		
Ethyl ester, homogeneous	-17.1	Methyl ester, homogeneous	+84		
<i>l</i> - $\alpha$ -Hydroxy- <i>n</i> -butyric acid		$\alpha$ -Bromo- <i>n</i> -butyric acid		<i>l</i> - $\alpha$ -Amino- <i>n</i> -butyric acid	
Ba salt in $H_2O$	-14.9	In $H_2O$	+24	In 20% HCl	+21.1
Acid in $H_2O$	+3.7	Na salt in $H_2O$	+3.5	" $H_2O$	+8.6
		In 30% alcohol	+57		
		" ether	+66		
		Homogeneous	+60		
Ethyl ester, homogeneous	-13	Methyl ester, homogeneous	+93		
<i>l</i> - $\alpha$ -Hydroxy- <i>n</i> -caproic acid		$\alpha$ -Bromo- <i>n</i> -caproic acid		<i>l</i> - $\alpha$ -Amino- <i>n</i> -caproic acid	
Ba salt in $H_2O$	-22.2	Na salt in $H_2O$	+25	In 20% HCl	+31.9
Na " "	-23	In 30% alcohol	+69		
Acid in $H_2O$	+5	" ether	+82		
		Homogeneous	+72		
Ethyl ester, homogeneous	-11	Methyl ester, homogeneous	+104		

—54.0°. By conversion of the first mother liquor of the brucine salt into the strychnine salt, we have been able to obtain the levorotatory form. The three  $\alpha$ -bromo acids discussed in this paper were resolved to the levorotatory acid through the strychnine salt (10).

*$\alpha$ -Bromocaproic acid* was resolved through the strychnine salt and the maximum rotation in homogeneous state was found to be  $[\text{M}]_D^{25} = -71.4^\circ$ .

Thus, again in the homologous series of  $\alpha$ -bromo acids rotating in the same direction the maximum values of the rotations of the successive members progressively increase in value.

*Series of  $\alpha$ -Amino Acids*—From Table I it may be seen that the rotations of the successive members of the series of  $\alpha$ -amino acids likewise increase in value. The correlation of the configurations of the individual members given in Table I was assumed on the basis of the method of their preparation. Every one of the acids can be prepared from the corresponding dextrorotatory  $\alpha$ -bromo acid.

In every case, the shift of the direction of rotation of every configurationally related member of each homologous series on passing from undissociated acid to the ion is in the same direction.<sup>1</sup>

The effect of temperature on the conversion of amino acids to hydroxy acids was studied, and it was found that there was not much more racemization at 90° than at 28°. At 0° this amounted to 24, 15, and 25 per cent, for the derivatives of propionic, butyric, and caproic acids respectively.

The racemization on conversion of the amino acid to the bromo acid with nitrosyl bromide is 15 per cent for the butyric acid and 22 per cent for the caproic acid.

The  $\alpha$ -hydroxybutyric acid, when treated with NO and Br<sub>2</sub>, was recovered unchanged.

<sup>1</sup> The levorotatory  $\alpha$ -bromo acids, which are produced by the action of nitrosyl bromide from amino acids possessing the *l* configuration, differ from the latter in that they become more dextrorotatory or less strongly levorotatory on ionization. Therein they also differ from the *l*- $\alpha$ -hydroxy acids produced by the action of nitrous acid upon *l*-amino acids. If this be taken as evidence that they possess the *d* configuration, it must be concluded that Walden inversion occurs during their formation by the reaction of  $\alpha$ -amino acids with nitrosyl bromide, and not during their conversion by ammonia into *d*-amino acids. (M. K.)

## EXPERIMENTAL

*d*(-)- $\alpha$ -Hydroxy-*n*-Butyric Acid—The preparation and resolution of the morphine salt was carried out in the manner described by Levene and Haller (4). The recrystallization of the morphine salt from 50 per cent alcohol was continued until a morphine salt was obtained which yielded a barium salt with  $[\alpha]_D^{20} = +8.6^\circ$  ( $c = 8.1$ ).

From the mother liquor from the last crystallization, a second crystalline deposit was obtained which yielded a barium salt with  $[\alpha]_D^{20} = +8.3^\circ$  ( $c = 10.3$ ).

The mother liquor of this second substance was converted into the barium salt, having  $[\alpha]_D^{20} = +8.0^\circ$  ( $c = 15.3$ ).

The crystalline morphine salts were combined and recrystallized four times from 50 per cent alcohol. Two fractions were obtained which on conversion into barium salts gave the following two fractions.

Fraction I.	$[\alpha]_D^{25} = +8.69^\circ$ ; $[M]_D^{25} = +14.9^\circ$ ( $c = 7.7$ )
“ II.	$[\alpha]_D^{25} = +8.50^\circ$ ; $[M]_D^{25} = +14.6^\circ$ (“ = 12.0)
	(Ba salt in water)

The  $[M]_D^{25} = +14.9^\circ$  may be accepted as the maximum rotation of the barium salt of *d*- $\alpha$ -hydroxy-*n*-butyric acid. Levene and Haller (4) report a rotation of  $[\alpha]_D^{24} = +7.7^\circ$  for it.

Fraction I was analyzed.

$C_8H_{14}O_6Ba$ .	Calculated.	C 27.95, H 4.11, Ba 39.99
	Found.	“ 27.47, “ 3.97, “ 39.93

*d*(+)- $\alpha$ -Hydroxy-*n*-Caproic Acid—For the resolution of this acid the procedure of Levene, Mori, and Mikeska (3) was followed. The cinchonidine salt was recrystallized from water until a sample of the crystals yielded a barium salt with  $[\alpha]_D^{25} = +10.6^\circ$  (in water) ( $c = 1.6$ ).

Part of the mother liquor of the above cinchonidine salt was converted into a barium salt having  $[\alpha]_D^{25} = +10.7^\circ$  ( $c = 1.7$ ).

The crystalline cinchonidine salt was recrystallized from water and a barium salt was obtained which had  $[\alpha]_D^{25} = +10.9^\circ$  ( $c = 1.7$ ).

The mother liquor yielded a barium salt with  $[\alpha]_D^{25} = +11.1^\circ$ ,

$[M]_D^{25} = +22.2^\circ$  ( $c = 1.7$ ), which was taken as the maximum value.

$C_{12}H_{22}O_6Ba$ . Calculated, Ba 34.38; found, Ba 34.44

Levene, Mori, and Mikeska (3) report  $[M]_D^{25} = +23^\circ$  for the sodium salt of  $\alpha$ -hydroxy-*n*-caproic acid. They give a value of  $[M]_D^{25} = -4.95^\circ$  for the free acid in  $H_2O$ .

*Rotations of (-)- $\alpha$ -Bromopropionic Acid (8)*—An acid of  $[\alpha]_D^{25} = -21.6^\circ$ ,  $[M]_D^{25} = -33.1^\circ$  (homogeneous), *i.e.* 75 per cent of the maximum, gave a rotation of  $[\alpha]_D^{25} = -28.1^\circ$ ,  $[M]_D^{25} = -43^\circ$  ( $c = 11.6$ ); whence the maximum  $[M]_D^{25} = -57^\circ$  in ether.

*Methyl  $\alpha$ -Bromopropionate (11)*—9.7 gm. of (-)- $\alpha$ -bromopropionic acid,  $[\alpha]_D^{25} = -21.6^\circ$  (homogeneous) = 75 per cent of the maximal value, were dissolved in 100 cc. of methanol containing 2 gm. of  $H_2SO_4$ . This was refluxed for 8 hours, concentrated to a small volume, and extracted with ether. The fraction boiling at  $41-43^\circ$ , 15 mm., was collected. Yield 3.4 gm.

$C_4H_7O_2Br$ . Calculated. C 28.75, H 4.23, Br 47.86

Found. " 29.30, " 4.33, " 46.97

$[\alpha]_D^{25} = -37.7^\circ$ ;  $[M]_D^{25} = -63.0^\circ$ ; maximum  $[M]_D^{25} = -84^\circ$  (homogeneous)

Walker (11) reports  $[\alpha]_D^{17} = +42.65^\circ$  for the dextrorotatory variety of this substance.

(+)- $\alpha$ -Bromo-*n*-Butyric Acid (3)—The resolution of the racemic acid was accomplished by extracting the brucine salt with acetone and with varying mixtures of acetone and chloroform. A typical example of the procedure is the following. The *dl*- $\alpha$ -bromo-*n*-butyric acid was dissolved in a mixture of 5 parts of acetone and 1 part of chloroform, and 1 equivalent of brucine was added. As soon as a crystalline precipitate formed (after several hours), it was filtered off. It is essential not to allow this mixture to stand overnight. The brucine salt was then extracted ten times with acetone at  $50^\circ$ . The operation was followed by extraction three times with a mixture of 9 parts of acetone and 1 part of chloroform at  $50^\circ$ ; then three times with a mixture of 8 parts of acetone to 2 parts of chloroform and finally four times with a mixture of 7 parts of acetone and 3 parts of chloroform. After each of the last three extractions portions were taken and converted into the free acids. The optical rotation,  $[\alpha]_D^{25} = +39.5^\circ$ ,  $[M]_D^{25} = +66.0^\circ$

(in ether) ( $c = 9.1$ ), was identical for each sample. Levene, Mori, and Mikeska (3) report  $[\text{M}]_{\text{D}}^{20} = +59^{\circ}$ .

The free acid was distilled. B.p.  $66\text{--}69^{\circ}$ , 0.04 mm.;  $d_4^{25} = 1.568$ .

$\text{C}_4\text{H}_7\text{O}_2\text{Br}$ . Calculated. C 28.75, H 4.23, Br 47.86

Found. " 28.86, " 4.19, " 47.96

$[\alpha]_{\text{D}}^{25} = +36.3^{\circ}$ ;  $[\text{M}]_{\text{D}}^{25} = +60.6^{\circ}$  ( $c = 9.5$ ); maximum  $[\text{M}]_{\text{D}}^{25} = +66^{\circ}$  (in ether)

$[\alpha]_{\text{D}}^{25} = +33.3^{\circ}$ ;  $[\text{M}]_{\text{D}}^{25} = +55.6^{\circ}$ ; maximum  $[\text{M}]_{\text{D}}^{25} = +60^{\circ}$  (homogeneous)

Thus, racemization to the extent of 8 per cent occurred during distillation.

About 1 year later the rotation of this same substance was again determined; it was 80 per cent of the maximal value, and thus had undergone a further racemization of 12 per cent. Its rotation was  $[\alpha]_{\text{D}}^{25} = +27.2^{\circ}$ ;  $[\text{M}]_{\text{D}}^{25} = +45.4^{\circ}$  ( $c = 9.1$ ); maximum  $[\text{M}]_{\text{D}}^{25} = +57^{\circ}$  (in 30 per cent alcohol).

*Methyl  $\alpha$ -Bromo-*n*-Butyrate*—10 gm. of (+)- $\alpha$ -bromo-*n*-butyric acid,  $[\alpha]_{\text{D}}^{25} = +26.5^{\circ}$  (homogeneous) = 74.3 per cent of the maximal value, were dissolved in 100 cc. of methanol containing 2 gm. of concentrated sulfuric acid and refluxed for 8 hours. The solution was then concentrated at  $25^{\circ}$  under reduced pressure to a small volume. The residue was extracted with ether, and the ether solution was washed with a 5 per cent solution of sodium carbonate, then with water, and dried over anhydrous sodium sulfate and distilled. Yield 7.4 gm.; b.p.  $57\text{--}59^{\circ}$ , 11 mm.;  $d_4^{25} = 1.402$ ;  $n_{\text{D}}^{25} = 1.4497$ .

$\text{C}_4\text{H}_9\text{O}_2\text{Br}$ . Calculated. C 33.15, H 5.01, Br 44.16

Found. " 33.16, " 4.99, " 44.21

$[\alpha]_{\text{D}}^{25} = +37.9^{\circ}$ ;  $[\text{M}]_{\text{D}}^{25} = +68.6^{\circ}$ ; maximum  $[\text{M}]_{\text{D}}^{25} = +93^{\circ}$  (homogeneous)

(-)- $\alpha$ -Bromo-*n*-Butyric Acid—The first mother liquor obtained from the brucine salt was liberated from the alkaloid and the free acid was converted into the strychnine salt. This was dissolved in 95 per cent alcohol at  $75^{\circ}$  and cooled rapidly. The crystalline deposit was recrystallized in the same manner. After eight crystallizations a sample of the acid was obtained which had  $[\alpha]_{\text{D}}^{25} = -31.7^{\circ}$ ;  $[\text{M}]_{\text{D}}^{25} = -57.4^{\circ}$  (in ether) ( $c = 14$ ), *i.e.* 87 per cent of the maximum. The resolution was not continued further.

(-)- $\alpha$ -Bromo-*n*-Caproic Acid<sup>2</sup>—The *dl*- $\alpha$ -bromo-*n*-caproic acid was prepared in the usual way by bromination of pure *n*-caproic acid. The acid (*cf.* (3)) was neutralized with a solution of strychnine in chloroform and the solution was concentrated under reduced pressure to a thick syrup. The residue was taken up in acetone and the crystalline precipitate was filtered off the same day. The precipitate was then suspended in acetone, and the mixture stirred well with a mechanical stirrer and then filtered. The operation was repeated several times. The bromo acid at this stage had  $[\alpha]_D^{25} = -24.5^\circ$  (in ether). The extraction was continued with boiling acetone, and after four extractions the rotation of the resulting acid reached the value of  $[\alpha]_D^{25} = -28.7^\circ$  (in ether). The further resolution was continued by recrystallization from a mixture of 8 parts of acetone and 2 parts of water. After the ninth crystallization the strychnine salt yielded an acid with a rotation of  $[\alpha]_D^{25} = -40.8^\circ$  (in ether). The eleventh recrystallization yielded an acid with the rotation of  $[\alpha]_D^{25} = -41.6^\circ$ ; the twelfth  $-42.2^\circ$ . After the thirteenth, it was  $[\alpha]_D^{25} = -42.1^\circ$  (in ether) (*c* = 36). Thus,  $[\alpha]_D^{25} = -42.2^\circ$ ,  $[M]_D^{25} = -82^\circ$  (in ether), was accepted as the maximum obtained by this manner of resolution. The acid distilled at 90–92°, 1 mm.;  $d_4^{25} = 1.367$ .

$C_6H_{11}O_2Br$ . Calculated, Br 40.98; found, Br 41.03

The rotation of the distilled product was  $[\alpha]_D^{25} = -36.2^\circ$ ,  $[M]_D^{25} = -70.6^\circ$ . This was 98.6 per cent of the maximal value, *i.e.* only 1.4 per cent racemization had occurred on distillation, for a sample of  $\alpha$ -bromo-*n*-caproic acid of  $[\alpha]_D^{25} = -34.0^\circ$  (in ether) (*c* = 17.4), *i.e.* 80.6 per cent of the maximal value, gave in the homogeneous state a rotation of  $[\alpha]_D^{25} = -29.6^\circ$ ,  $[M]_D^{25} = -57.7^\circ$ , *i.e.* 80.6 per cent of the maximal value. Maximum  $[M]_D^{25} = -71.6^\circ$  (homogeneous).

From existing data (3) the following rotations were calculated, with  $[\alpha]_D^{25} = -42.2^\circ$  (in ether) as the maximum. In 30 per cent alcohol  $[\alpha]_D^{25} = -35.2^\circ$ ;  $[M]_D^{25} = -68.6^\circ$  (maximum); Na salt in water  $[\alpha]_D^{25} = -11.3^\circ$ ;  $[M]_D^{25} = -24.5^\circ$  (maximum).

(-)-Methyl  $\alpha$ -Bromo-*n*-Caproate—10 gm. of (-)- $\alpha$ -bromo-*n*-

<sup>2</sup> Levene and Mardashew (12) report  $[\alpha]_D^{20} = -37.97^\circ$  for  $\alpha$ -bromo-*n*-caproic acid resolved in this manner. This value should have read  $\alpha_D^{20} = -37.97^\circ$  (homogeneous, 1 dm.).



caproic acid,  $[\alpha]_D^{25} = -36.2^\circ$  (homogeneous) = 98.6 per cent of the maximal value, were refluxed for 8 hours with 100 cc. of methanol containing 2 gm. of concentrated sulfuric acid. The solution was concentrated to 15 cc. at  $25^\circ$  under reduced pressure. The residue was extracted with ether, and the ethereal solution washed with a 5 per cent sodium carbonate solution and then with water and dried over anhydrous sodium sulfate. The fraction boiling at  $60-61^\circ$ , 1 mm., was collected. The yield was 6.4 gm.;  $d_4^{25} = 1.282$ ;  $n_D^{25} = 1.4520$ .

$C_7H_{13}O_2Br$ . Calculated. C 40.18, H 6.28, Br 38.23

Found. " 40.30, " 6.41, " 38.28

$[\alpha]_D^{25} = -49.4^\circ$ ;  $[M]_D^{25} = -103^\circ$ ; maximum  $[M]_D^{25} = -104^\circ$  (homogeneous)

*Synthesis of l(+)- $\alpha$ -Amino-n-Butyric and of l(+)- $\alpha$ -Amino-n-Caproic Acids*

The synthesis was accomplished by a modification of the usual method (13) which led to yields of over 85 per cent for aminobutyric and 92.5 per cent for aminocaproic acid. There was practically no racemization in the course of the reaction. Hence, for the preparation of optically active amino acids in cases in which the acid can be prepared by the action of ammonia, it is preferable to resolve the bromo acid rather than the amino acid. It is not necessary to resolve the bromo acid to the maximum rotation, for the  $\alpha$ -amino acids containing about 20 per cent of the *dl* form are readily purified to maximum rotation by two or three recrystallizations from water.

A typical example of the preparation of *l*(+)- $\alpha$ -aminobutyric acid is the following. 13 gm. of (+)- $\alpha$ -bromo-*n*-butyric acid,  $[\alpha]_D^{25} = +26.5^\circ$  (homogeneous) = 74.3 per cent of the maximal value, were slowly added to 100 cc. of saturated aqueous ammonia. The solution was allowed to stand at room temperature for 2 days and then evaporated to complete dryness under reduced pressure. To remove residual moisture, small portions of methanol and of benzene were added to the residue and the solvents were removed by distillation under reduced pressure at  $40-50^\circ$ . The operation was repeated several times. The residue was then extracted with hot methanol and the operation continued until the residue no longer showed the presence of bromide ions. As a rule two or three extractions sufficed. The extracts were again

concentrated to dryness under reduced pressure and the residue was again extracted with hot methanol. If an insoluble residue was obtained, the operation of concentrating the mother liquor with subsequent extraction was continued until the product was completely soluble in methanol. In this experiment the yield of the first residue was 6 gm. and from the extracts an additional 10 gm. were obtained, a total yield of 87 per cent of amino acid.  $[\alpha]_D^{25} = +16.0^\circ$  ( $c = 5.0$ ) (in 20 per cent HCl), corresponding to 78 per cent of the maximal value (see below). Though the starting material contained 25.7 per cent of the *dl* form, the amino acid contained only about 22 per cent of the *dl* form.

$C_4H_9O_2N$ . Calculated, N 13.58; found, N 13.76

This substance was recrystallized five times from water, when its rotation in 20 per cent HCl was  $[\alpha]_D^{25} = +20.5^\circ$  ( $c = 10$ ), maximum  $[M]_D^{25} = +21.1^\circ$  (in 20 per cent HCl).

*d*(-)- $\alpha$ -Amino-*n*-Caproic Acid (12)---55 gm. of (-)- $\alpha$ -bromo-*n*-caproic acid,  $[\alpha]_D^{25} = -32.3^\circ$  (homogeneous) = 88 per cent of the maximal value, were dissolved in 565 cc. of saturated aqueous ammonia and allowed to stand at room temperature for 36 hours. The further procedure was as above. The residue obtained on concentration of the first methanol extract was completely soluble in hot methanol. The first residue insoluble in hot methanol was washed on a Buchner funnel with methanol until free from bromide ions. The yield was 34 gm. or 92.5 per cent.

$C_6H_{13}O_2N$ . Calculated. C 54.92, H 10.00,  $NH_2-N$  10.69

Found. " 54.94, " 9.99, " 10.67

$[\alpha]_D^{25} = -21.4^\circ$ ,  $[M]_D^{25} = -28^\circ$  (in 20 % HCl) ( $c = 9.4$ )

Marko (14) reports  $[\alpha]_D^{20} = +23.1^\circ$  (in 20 per cent HCl,  $c = 4.6$ ),  $[M]_D^{20} = +30^\circ$ , which can be considered maximum.

*Action of  $HNO_2$  on Alanine at Different Temperatures.* At  $28^\circ$  in Aqueous Solution---10 gm. of *l*(+)-alanine,  $[\alpha]_D^{20} = +7.10^\circ$  (in 20 per cent HCl) = 46 per cent of the maximal value, were dissolved in 124 cc. of *N* sulfuric acid and a solution of 8.6 gm. of  $NaNO_2$  in 25 cc. of water was added at a slow rate in the course of 10 minutes with constant stirring. At the end of this period another 25 cc. of an identical solution were added in the same manner. The reaction product was then neutralized with sodium

hydroxide and the solution was concentrated to dryness. To remove traces of water a mixture of benzene and alcohol was added and removed by distillation under reduced pressure, the operation being repeated several times. The salts were then esterified with ethanol in the usual way and the esters fractionated. The fraction boiling at 146–153° was collected.

The rotation of the ethyl *l*-lactate was  $[\alpha]_D^{20} = -4.06^\circ$  (homogeneous), *i.e.* 28 per cent of the maximal value (see below); racemization = 39 per cent.

*At 0° in Aqueous Solution*—10 gm. of *l*(+)-alanine,  $[\alpha]_D^{20} = +7.10^\circ$  (in 20 per cent HCl) = 46 per cent of the maximal value, were dissolved in 124 cc. of *N* sulfuric acid, cooled in an ice water bath, and a solution of 8.6 gm. of  $\text{NaNO}_2$  in 50 cc. of water was added in the course of 45 minutes with constant stirring. At the end of this period another 8.6 gm. of  $\text{NaNO}_2$  were added in the same manner. 2 cc. of the solution at this time when analyzed for amino nitrogen gave 1.4 cc. of  $\text{N}_2$  (Van Slyke). Another 8.6 gm. portion of  $\text{NaNO}_2$  was added and the solution was stirred in the ice bath for an additional hour. 2 cc. of the solution then gave 0.2 cc. of  $\text{N}_2$ . The entire reaction required  $3\frac{1}{4}$  hours. The solution was neutralized with alkali and concentrated to a small volume. Then it was acidified with 40 per cent phosphoric acid and extracted with ether. The ether extract was dried with anhydrous sodium sulfate and evaporated to a small volume. Barium hydroxide solution was added in excess. The solution was neutralized with sulfuric acid and centrifuged. The supernatant liquid was concentrated to dryness and the barium salt dried. The weight of barium salt was 9.5 gm. This was esterified with ethanol in the usual way, and the ester fractionated. The ethyl *l*-lactate, b.p. 148–153°, had  $[\alpha]_D^{20} = -5.05^\circ$ ,  $[\text{M}]_D^{20} = -5.96^\circ$  (homogeneous), *i.e.* 35 per cent of the maximal value; maximum  $[\text{M}]_D^{19} = -17.1^\circ$  (11). Thus 24 per cent racemization took place during the reactions.

*At 90° in Glacial Acetic Acid*—10 gm. of *l*(+)-alanine,  $[\alpha]_D^{20} = +7.10^\circ$  (in 20 per cent HCl), were dissolved in 300 cc. of glacial acetic acid and heated to 90° on a boiling water bath and 17 gm. of pulverized  $\text{NaNO}_2$  were added in small portions with constant stirring. Further treatment was the same as in the previous experiment.

The resulting ethyl *l*-lactate had  $[\alpha]_D^{20} = -3.79^\circ$  (homogeneous) = 26 per cent of the maximal value; whence racemization = 43 per cent.

$C_5H_{10}O_3$ . Calculated. C 50.81, H 8.54  
Found. " 50.20, " 8.56

*Action of NOBr on Alanine*—An aqueous solution of 5 gm. of *l*(+)-alanine,  $[\alpha]_D^{25} = +14.7^\circ$  (in 20 per cent HCl), *i.e.* 96 per cent of the maximal value, was cooled in ice and an ethereal solution of nitrosyl bromide was added until the yellow color persisted for some time. The mixture was allowed to stand at room temperature for  $1\frac{1}{2}$  hours. The bromopropionic acid was then extracted with ether and purified by fractional distillation.

$C_3H_5O_2Br$ . Calculated. C 23.54, H 3.29  
Found. " 23.21, " 3.10

$[\alpha]_D^{25} = -20.2^\circ$ ,  $[M]_D^{25} = -31.4^\circ$  (homogeneous) = 71.5 per cent of the maximum; 25.6 per cent racemization.

Levene and Haller (4) established the relationship between the barium salt and the ethyl ester of  $\alpha$ -hydroxy-*n*-butyric acid. From a barium salt of  $[\alpha]_D^{24} = +7.7^\circ$ ,  $[M]_D^{24} = +13.2^\circ$ , an ethyl ester was obtained with  $[\alpha]_D^{22} = +8.59^\circ$ ,  $[M]_D^{22} = +11.3^\circ$  (homogeneous). This salt gave a free acid of  $[\alpha]_D^{22} = -3.2^\circ$  (calculated) (in  $H_2O$ ). The least maximum rotations, with  $[M]_D^{25} = +14.9^\circ$  for the barium salt, are as follows: for the ethyl ester  $[M]_D^{25} = +12.8^\circ$  (homogeneous) and for the free acid  $[M]_D^{25} = -3.7^\circ$  (in  $H_2O$ ).

*Action of NOBr on  $\alpha$ -Hydroxybutyric Acid*—This experiment aimed at ascertaining whether in the substitution of  $NH_2$  by Br by reaction with NOBr the formation of the hydroxy acid is not the prior reaction, followed by its bromination. 13 gm. of the barium salt of the *dl*- $\alpha$ -hydroxy-*n*-butyric acid were suspended in 10 cc. of water and 32.5 cc. of 40 per cent hydrobromic acid. 5 cc. of bromine were added to the solution and nitric oxide was passed through the solution which was kept in a freezing mixture of ice and salt for 3 hours. At the end of that time 2 cc. of bromine were again added. The gas was passed during an additional 2 hours. The bromine was removed in the usual way and the reaction product extracted with ether. The acid distilled at 138–

144° at 15 mm. pressure and the distillate crystallized on standing. It contained no bromine.

$C_4H_7O_3$ . Calculated. C 46.13, H 8.75  
Found. " 45.67, " 7.80

*Action of  $HNO_2$  on  $l(+)$ - $\alpha$ -Amino- $n$ -Butyric Acid*—20 gm. of  $\alpha$ -amino- $n$ -butyric acid,  $[\alpha]_D^{25} = +10^\circ$  (in 20 per cent HCl), *i.e.* 49 per cent of the maximal value, were converted into the hydroxy acid at  $0^\circ$  in aqueous solution in the manner described in the case of alanine. The reaction product was converted into the ethyl ester. It boiled at 49–57° at 15 mm. The yield was 10 gm. of ethyl  $l$ - $\alpha$ -hydroxybutyrate.

$C_6H_{12}O_3$ . Calculated. C 54.40, H 9.16  
Found. " 54.42, " 9.11

$[\alpha]_D^{25} = -4.0^\circ$  (homogeneous),  $[M]_D^{25} = -5.3^\circ = 41.4$  per cent of the maximal value of  $[M]_D^{25} = -12.8^\circ$ . Thus 15 per cent racemization took place.

In the same way, 10 gm. of  $l(+)$ - $\alpha$ -amino- $n$ -butyric acid,  $[\alpha]_D^{25} = +17.3^\circ$  (in 20 per cent HCl), *i.e.* 85 per cent of the maximal value, were deaminized at  $0^\circ$  and the hydroxy acid was converted into the barium salt. The yield was 15 gm.

$C_8H_{14}O_6Ba$ . Calculated. C 27.95, H 4.11, Ba 39.99  
Found. " 27.27, " 4.11, " 40.53

$[\alpha]_D^{25} = -6.29^\circ$ ,  $[M]_D^{25} = -10.8^\circ$  (in  $H_2O$ ) ( $c = 10.5$ ); *i.e.*, 72.5 per cent of the maximum  $[M]_D^{25} = -14.9^\circ$ . Thus 15 per cent of racemization took place during the reaction.

*Action of  $NOBr$  on  $\alpha$ -Amino- $n$ -Butyric Acid*—10 gm. of  $l(+)$ - $\alpha$ -amino- $n$ -butyric acid,  $[\alpha]_D^{25} = +7.5^\circ$  (in 20 per cent HCl), *i.e.* 37 per cent of the maximal value, were treated in the manner described in the case of  $\alpha$ -hydroxy- $n$ -butyric acid. 13 gm. of the bromo acid were obtained, which boiled at 103–108°, 15 mm.

$C_4H_7O_2Br$ . Calculated, Br 47.9; found, Br 47.86

The rotation of the  $\alpha$ -bromobutyric acid was  $[\alpha]_D^{23} = -11.7^\circ$  (homogeneous), *i.e.* 32 per cent of the maximum, which corresponds to 13.5 per cent racemization in the course of the reaction.

*$d(-)$ - $\alpha$ -Hydroxy- $n$ -Caproic Acid*—15 gm. of  $d(-)$ - $\alpha$ -amino-

*n*-caproic acid,  $[\alpha]_D^{25} = -21.4^\circ$  (in 20 per cent HCl) = 88 per cent of the maximal value, were deaminized at  $0^\circ$  in aqueous solution in the manner described above. The barium salt was isolated.

$C_{12}H_{22}O_6Ba$ . Calculated, Ba 34.38; found, Ba 34.45

$[\alpha]_D^{24} = +7.31^\circ$ ,  $[M]_D^{24} = +14.4^\circ$  (in  $H_2O$ ), which is 65 per cent of the maximum of  $[M]_D^{25} = -22.2^\circ$ . There is 25 per cent racemization.

(+)- $\alpha$ -Bromo-*n*-Caproic Acid—10 gm. of *d*(-)- $\alpha$ -amino-*n*-caproic acid,  $[\alpha]_D^{25} = -21.4^\circ$  (in 20 per cent HCl) = 88 per cent of the maximal value, were treated with NOBr in aqueous solution in the manner described above. The yield of (+)- $\alpha$ -bromocaproic acid was 5 gm. It distilled at  $86-89^\circ$ , 0.35 mm.

$C_6H_{11}O_2Br$ . Calculated. C 36.92, H 5.69, Br 40.98  
Found. " 37.09, " 5.74, " 40.60

$[\alpha]_D^{25} = +25.3^\circ$ ,  $[M]_D^{25} = +49.5^\circ$  (homogeneous), which is 69 per cent of the maximal value; the racemization therefore was 22 per cent.

Ethyl *d*(+)- $\alpha$ -Hydroxy-*n*-Caproate 9 gm. of barium *d*(+)- $\alpha$ -hydroxy-*n*-caproate,  $[\alpha]_D^{25} = +7.31^\circ$  (in  $H_2O$ ) = 66 per cent of the maximal value, were suspended in 18 cc. of absolute alcohol and 2.6 gm. of concentrated sulfuric acid in 7 cc. of absolute alcohol were slowly added with shaking. This was refluxed overnight. The ethyl *d*(+)- $\alpha$ -hydroxycaproate, isolated as usual, distilled at  $49-53^\circ$ , 0.6 mm. The yield was 2.6 gm.;  $d_4^{25} = 0.9664$ .

$C_8H_{16}O_3$ . Calculated. C 59.95, H 10.07  
Found. " 59.95, " 9.91

$[\alpha]_D^{25} = +4.68^\circ$ ,  $[M]_D^{25} = +7.5^\circ$ ; whence the least maximum  $[M]_D^{25} = +11^\circ$  (homogeneous).

#### SUMMARY

1.  $\alpha$ -Hydroxy-*n*-butyric and  $\alpha$ -hydroxy-*n*-caproic acids were resolved to maximal rotation. The molecular rotations of the barium salts of the *l* acids are  $-14.9^\circ$  and  $-22^\circ$  respectively.

2. The rotation of  $\alpha$ -bromopropionic acid was determined in ether, and it was converted into the methyl ester. The maximum rotation in ether was found to be  $[M]_D^{25} = -57^\circ$ , and that of its ester  $[M]_D^{25} = -84^\circ$ .

3.  $\alpha$ -Bromo-*n*-butyric and  $\alpha$ -bromo-*n*-caproic acids were resolved to maximum values of  $[M]_D^{25} = +60^\circ$  and  $-72^\circ$  respectively. Their methyl esters had  $[M]_D^{25} = +92^\circ$  and  $-104^\circ$ .

4.  $\alpha$ -Amino-*n*-butyric and  $\alpha$ -amino-*n*-caproic acids were obtained from the  $\alpha$ -bromo acids, and maximum rotations of  $[M]_D^{25} = +21.1^\circ$  and  $-31.9^\circ$  respectively were obtained.

5. Alanine was treated with nitrous acid at  $0^\circ$ ,  $28^\circ$ , and  $90^\circ$  and converted to the ethyl ester; the racemization was found to be 24, 39, and 43 per cent respectively.

6.  $\alpha$ -Amino-*n*-butyric acid was treated with nitrous acid at  $0^\circ$  and converted into the ethyl ester, and the racemization was found to be 15 per cent.

7.  $\alpha$ -Amino-*n*-butyric and  $\alpha$ -amino-*n*-caproic acids were treated with nitrous acid at  $0^\circ$  and the hydroxy acids were converted into the barium salts. The racemization was found to be 15 per cent and 25 per cent respectively.

8. Alanine,  $\alpha$ -amino-*n*-butyric and  $\alpha$ -amino-*n*-caproic acids were converted into their respective bromo acids and the racemization was found to be 25.6, 13.5, and 22 per cent.

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## STUDIES ON THE RESPIRATORY PIGMENT OF URECHIS EGGS

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Experiments previously reported (1) have shown that the eggs of the Pacific marine worm, *Urechis caupo*, contain a reversible oxidation-reduction pigment. The pigment, called *urechrome*, is autoxidizable and changes color from red to yellow on oxidation. It is soluble in water (reduced form insoluble below pH 5) and in acidified methanol, but insoluble in ether, acetone, chloroform, and neutral alcohol. Evidence for participation of the pigment in cellular respiration has been previously given.

Because of its biochemical interest and possible embryological significance, a more extensive study of the substance has been made, the results of which are reported below.

*Occurrence and Preparation*—Urechrome occurs chiefly in the eggs of the worm. None has been found in the muscle, viscera, egg sacs, or sperm. Traces are found in the centrifuged blood elements of the female, probably in association with the oocytes, which mature in the blood of this species.

To prepare the pigment, the egg sacs are dissected from the animal and dropped into sea water, care being taken to prevent contamination by blood. The eggs are released by puncturing the sacs and the empty sacs are removed. After the eggs have been washed several times, they are centrifuged down and transferred to a Soxhlet apparatus where they are extracted first with acetone and then with ether until yellow pigments no longer come over. The urechrome, which remains behind, is extracted by shaking with 0.1 N HCl-methanol at 40°. The solution is

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filtered and then concentrated *in vacuo* to about one-fourth of the original volume. It is now brought to pH 2 (glass electrode) with 1 N NaOH, precipitating reduced urechrome. After standing at 0° for several hours, the precipitate is centrifuged down, redissolved in HCl-methanol, and reprecipitated with 4 to 5 volumes of ether. The ether precipitation is repeated, after which the pigment is collected in a centrifuge tube, washed twice with absolute methanol, and twice with distilled water. It is dried *in vacuo* over calcium chloride. The reduced pigment dries in dark violet, glistening flakes. It is non-crystalline. 10 gm. of dehydrated, defatted eggs yield about 40 to 50 mg. of urechrome. An additional 15 to 20 mg. can be recovered from the supernatant of the NaOH precipitation by reducing the oxidized pigment, which has remained in solution, with a small crystal of stannous chloride and proceeding as with the first precipitate. It has been found that reduced urechrome can also be precipitated from HCl-methanol with a small amount of pyridine, apparently as the result of salt formation.

#### EXPERIMENTAL

*Oxidation-Reduction Potential*—The oxidation-reduction potential has been studied polarographically (2-4), checked by potentiometric titration. The advantage of the polarographic method consists in its being particularly adaptable to small quantities in which the ratios of oxidant to reductant and the corresponding values of  $E_h$  are determinable without the addition of a second system. The method as used by us is less accurate than the potentiometric titration, the error being  $\pm 5$  millivolts.

The instrument used was the Heyrovsky-Nejedly type with a dropping mercury electrode and a saturated calomel reference half-cell. The set-up was essentially that described by Müller and Baumberger (2). The temperature was  $25^\circ \pm 1^\circ$ .<sup>1</sup> A saturated solution of pigment (1 to 3 mg. per cc.) in phosphate or borate buffer of the desired pH was deoxygenated by a stream of O<sub>2</sub>-free nitrogen. A small quantity of platinized asbestos was added and the gas changed to hydrogen. At various stages of reduction (judged roughly by the color of the solution) the hydro-

<sup>1</sup> Owing to a typographical error, the temperature of the experiments was previously reported as 250° (1).

gen was replaced by nitrogen and a current-voltage curve was photographically recorded (Fig. 1). After complete reduction had been attained, the process could be reversed by introducing successive small amounts of air into the solution until complete oxidation was reached. From the polarographic curves so obtained,  $E_h$  and the relative heights of the cathodic and anodic waves can be measured, from which  $E'_0$ , the ratio  $[\text{Ox}]:[\text{Red}]$ , and the number of electrons involved in the reaction can be calculated.

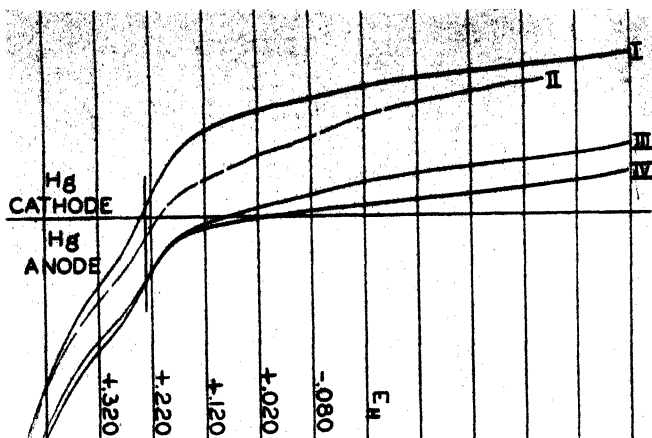


FIG. 1. Polarogram of urechrome. Phosphate buffer, pH 6.26. The horizontal line indicates "galvanometer zero," above which a dropping Hg electrode is the cathode and below which it is the anode. A short vertical line is drawn through the half waves, intersecting "galvanometer zero" at  $E'_0$ . The ratios  $[\text{Ox}]:[\text{Red}]$  are as follows: Curve I, 4:3; Curve II, 7:18; Curves III and IV, practically zero.

Details of the calculations are given in the paper of Müller and Baumberger (2). The potentials were readily reproducible, and were not noticeably affected by variations in the total concentration of dye.

The change in  $E'_0$  with pH was investigated from pH 5 to 10. These are the practical limits of pH for the substance, since below pH 5 the reduced form is insoluble and above pH 10 the pigment is unstable.  $E'_0$  decreases by 59 millivolts per unit increase in pH, within the limits of experimental error (Table I).

The reduction of urechrome involves only 1 electron, as can

be seen in Table I, where calculated values of  $E_h$  for 1 and 2 electron processes are compared with the observed potentials. With some preparations a second polarographic wave was observed at a potential several hundred millivolts negative to the main wave. The height and slope of this wave were extremely variable, in some cases being pronounced, in others imperceptible. The

TABLE I  
*Relation of  $E'_0$  to pH and Evaluation of  $n$ , from Polarographic Data*

pH	$E'_0$	$\frac{[\text{Ox}]}{[\text{Red}]}$	$E_h$		
			Calculated $n = 1$	Calculated $n = 2$	Observed
			volt	volt	volt
5.31	0.287				
6.26	0.228	4:3	0.236	0.232	0.239
		7:18	0.203	0.215	0.206
7.39	0.163	3:1	0.192	0.178	0.191
		9:7	0.170	0.167	0.172
		5:36	0.112	0.137	0.106
		2:25	0.097	0.130	0.091
		1:23	0.081	0.122	0.077
7.70	0.143*				
8.62	0.089*				
9.15	0.057	7:3	0.079	0.068	0.077
		19:13	0.067	0.062	0.066
		21:16	0.064	0.061	0.063
		2:11	0.013	0.035	0.011
		8:47	0.011	0.034	0.011
		1:23	-0.025	0.016	-0.020
9.97	0.007	7:1	0.058	0.033	0.056
Mean deviation from observed....			0.003	0.019	

\* Potentiometric titration.

"half wave" potential did not vary in any regular manner with pH, nor was it ever observed to fall below the "galvanometer zero" line, *i.e.* in the region corresponding to a completely reduced system, even after prolonged bubbling of hydrogen. We believe the wave to be due to an unknown impurity or decomposition product.

As a check on the polarographically determined potentials a few potentiometric titrations with a platinum electrode were carried

out. The reducing agent was  $\text{Na}_2\text{S}_2\text{O}_4$ , the oxidizing agent ferricyanide. The atmosphere was nitrogen. The results on the whole are in close agreement with the polarographically determined potentials, although the curves tend to be somewhat steeper than the theoretical, with drifting potentials at the extremes of the curve. This is attributed to poor poisoning of the system. A titration curve at pH 8.62 is shown in Fig. 2. The agreement between the polarographic and titrimetric measure-

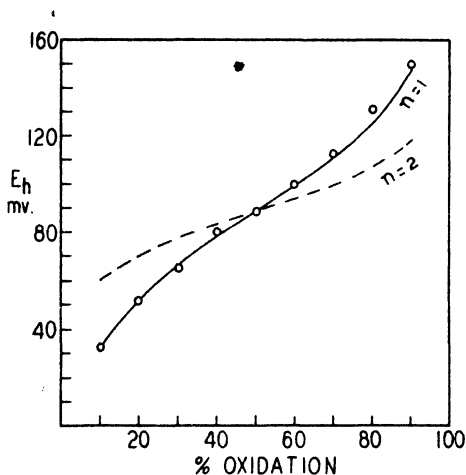


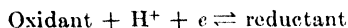
FIG. 2. Titration of reduced urechrome with ferricyanide, pH 8.62. The curves are the theoretical for  $n = 1$  and  $n = 2$ . The circles represent experimental values.

ments is in accord with the findings of previous studies on electro-active reversible systems.

We conclude that urechrome is a reversible oxidation-reduction system conforming, within the experimental limits, to the equation

$$E_h = 0.599 + \frac{RT}{F} \ln \frac{[\text{Ox}]}{[\text{Red}]} + \frac{RT}{F} \ln [\text{H}^+]$$

where the formulation of the reaction is as follows:



**Absorption Spectrum**—Solutions of the pigment exhibit no specific absorption in the visible region. Aqueous solutions of the

oxidized form show general absorption of wave-lengths below 540  $m\mu$ , whereas the reduced form absorbs generally below 620  $m\mu$ . Reduced urechrome dissolved in acid methanol shows a band in the near ultraviolet at 388  $m\mu$ . The oxidized form has not been investigated in this region.

Reduced urechrome forms a deep violet solution in concentrated sulfuric acid. This solution exhibits two marked absorption bands: a broad band centering at 553  $m\mu$  and a narrow band at 688  $m\mu$ . The bands resemble those of hematoporphyrin anhydride, formed from hemoglobin in concentrated sulfuric acid, which shows a broad band at 553  $m\mu$  and a narrow one at 613  $m\mu$ . It was therefore attempted to make a pyridine hemochromogen derivative of urechrome by reduction in alkaline solution in the presence of pyridine. The resulting solution showed a strong band at 548  $m\mu$  and a weak band at 515  $m\mu$ , typical of pyridine hemochromogen. It should be noted that the spectrum of pyridine hemochromogen cannot have resulted from contamination of the preparation with hemoglobin, since no hemoglobin spectrum was observed in untreated solutions, nor is the spectrum obtained by sulfuric acid treatment that of hematoporphyrin.

*Molecular Weight*—Since the degree of purity of the pigment was not known, the molecular weight was approximated from diffusion measurements. A Northrop and Anson type of diffusion cell (5, 6) of 7.29 cc. capacity was used. The membrane constant,  $K$ , determined by standardization against 2 M NaCl, was 0.470. The temperature of the thermostat was  $20^\circ \pm 0.05^\circ$ . In order to obtain a high concentration of urechrome in the diffusion cell, acidified methanol was the solvent used in the dye experiments. Relative concentrations on the two sides of the membrane were measured with an Evelyn photoelectric colorimeter.

Three determinations of the diffusion constant of urechrome gave  $D_{20, \text{methanol}} = 0.363, 0.366, \text{ and } 0.377$  sq. cm. per day; average, 0.368. Assigning the values 0.0062 to the viscosity of the solvent and 1.3 to the density of the pigment, one arrives at an approximate molecular weight of 1700. The method of calculation is given by Northrop and Anson (5). Since the calculation is based on the assumptions of a spherical molecule and the absence of solvation and ionization effects, the strict validity of which is in most cases doubtful, this value is to be taken as an approximation only.

The molecular weight from diffusion data is in fair agreement with that calculated from hydrogenation experiments. Urechrome is rapidly and reversibly reduced by hydrogen gas in the presence of finely divided platinum. From the potentiometric data it is known that the reduction involves 1 hydrogen atom per molecule of pigment. By carrying out the reaction in Warburg manometers, the uptake of hydrogen can be measured and the equivalent weight of pigment calculated. It was found that 8.18 c.mm. of  $H_2$  are taken up per mg. of pigment. The weight of pigment equivalent to 1 gm. atom of hydrogen is then

$$\frac{\frac{1}{2} \times 22,400}{8.18} = 1370$$

#### DISCUSSION

Urechrome is an autoxidizable pigment of relatively high potential. Evidence for its participation in the respiration of the egg cell has been presented in a previous paper. Because of the difficulty of obtaining sufficient quantities of the substance, further chemical studies of the molecule have not been possible. The available data, however, suggest that it may be related to the heme pigments. Such a hypothesis is consistent with the observed valence change of 1 on reduction, with the absorption spectra of the derivatives studied, and with the solubility properties of the pigment. That it is not a typical heme pigment, however, is evidenced by the absence of characteristic absorption bands in solutions of the untreated dye, as well as by the molecular weight, which appears to be too high for that of a free metalloporphyrin and too low for that of a chromoprotein. The fact that the pigment is not extracted from the cell by aqueous solvents, although it is soluble in them, suggests that, as obtained in extracts, it may represent a fragment of a larger molecule. This would explain the molecular weight and possibly other properties of the substance.

Concerning the biological significance of urechrome, the outstanding fact is that it has been found only in the eggs. Its potential, which is in the range of the cytochromes, and its autoxidizability indicate that it intermediates between oxygen and the reducing systems of the cell. It is noteworthy that spectroscopic examinations made by us of both living eggs and extracts have failed to

reveal the absorption bands of cytochrome. The studies of Brachet (7) and of Ball and Meyerhof (8) on the eggs of other species have given similar results. As is pointed out by the latter authors, this does not constitute proof that the cytochromes are not present, since they may occur in quantities too small for detection by ordinary methods. They suggest the possibility that a hemin other than cytochrome may be involved in the respiration of the egg. The present work constitutes evidence that such may be the case in the eggs of *Urechis*. On the basis of microscopic examinations, Baumberger and Michaelis (9) have concluded that pigmented granules present in these eggs are derived from the hematin of the blood. It appears likely that these granules are to be identified with urechrome, although this would be difficult to prove. It has been observed (10) that during the embryonic development of the egg red granules become localized in the ciliated regions of the embryo, suggesting a relationship with the ciliary metabolism.

#### SUMMARY

1. Urechrome, the red pigment of the eggs of *Urechis caupo*, is a reversible oxidation-reduction system. At pH 7  $E'_0 = +0.186$  volt;  $n = 1$ .

2. Solutions of the untreated pigment show no specific absorption in the visible region. In concentrated sulfuric acid bands appear at 553 and 688  $m\mu$ . Reduction in alkaline pyridine solution brings out the spectrum of a pyridine hemochromogen.

3. The approximate molecular weight determined from diffusion experiments is 1700.

4. It is suggested that the pigment is related to the hemins.

5. The possible physiological rôle of urechrome is discussed.

One of us (N. H. H.) desires to express his gratitude to Professor D. M. Whitaker and Professor C. B. van Niel of Stanford University and the Hopkins Marine Station for their generous aid during his residence there.

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# THE OCCURRENCE AND RATE OF TURNOVER OF TUMOR SPHINGOMYELIN\*

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While the rate of turnover of the total phospholipids of neoplastic tissue has been investigated by the use of radioactive phosphorus (7, 10), that of the individual phospholipids has received but limited attention. Haven (8) found that the lecithin fraction of Carcinosarcoma 256 had a more rapid rate of turnover than the cephalin fraction. Although sphingomyelin is known to occur in the active fraction of chicken tumor (4), it has not been reported present in mammalian tumors. Accordingly this investigation was undertaken to determine (a) to what extent sphingomyelin occurred in rat Carcinosarcoma 256 and (b) its rate of turnover as measured by radioactive phosphorus.

## EXPERIMENTAL

*Tumor*—Carcinosarcoma 256 was used throughout these experiments. The rats used and the method of inoculation have been previously described (8). The tumors were from 4 to 11 weeks old at the time of administration of radioactive phosphorus.

*Preparation and Administration of Disodium Hydrogen Phosphate Containing Radioactive Phosphorus*—Red phosphorus which had been made radioactive in the University of Rochester cyclotron was dissolved in concentrated nitric acid, filtered, and carefully taken almost to dryness several times. After final oxidation with aqua regia, the solution was concentrated nearly to dryness

\* The substance of this paper was presented before the American Society for Experimental Pathology at Chicago, April, 1941 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **140**, p. cxlv (1941)). This investigation was aided by grants from the International Cancer Research Foundation and the Rockefeller Foundation.

and the residue dissolved in water; after neutralization with sodium carbonate to a pH of 6 to 7, the solution was made to volume.

Each tumor-bearing rat received 1 cc. of the above solution containing approximately 100,000 counts per minute on our scale-of-four Geiger-Müller counter. The rats in Series I received the phosphate solution by stomach tube followed by 30 drops of cod liver oil; those in Series II received the solution subcutaneously in the interscapular region of the back.

*Treatment of Tissue and Extraction of Lipids*—From 6 hours to 6 days after administration of the radioactive phosphorus the animals were killed by decapitation and the tumors removed. In Series I the whole tumor was used for analysis; in Series II the tumor was separated grossly into peripheral and central tissue. The fresh tissue was ground with sand, and the lipids were extracted with various solvents. In a preliminary series of experiments the solvents used were alcohol-ether (3:1) followed by ether. Since it was found that the sphingomyelin was not completely extracted by the use of these solvents alone, in all subsequent experiments chloroform-methanol (1:1) was used in addition.

*Isolation of Sphingomyelin As Reineckate*—The solvent was removed from the lipid extracts in a partial vacuum under nitrogen with the addition of absolute alcohol toward the end of the distillation. After the residue was thoroughly extracted with chloroform, the solution was filtered and made to volume. An aliquot was taken for determination of total lipid phosphorus by the method of Kuttner and Lichtenstein (11) and of radioactivity by the method of Bale, Haven, and LeFevre (1). Duplicate aliquots of the remaining solution were taken for isolation of sphingomyelin as the reineckate. The procedure used was essentially that of Thannhauser and Setz (15), later modified by Thannhauser, Benotti, and Reinstein (12) and recently used by Erickson, Avrin, Teague, and Williams (5). Modifications of Thannhauser's method had already been worked out in this laboratory by Hunter (9) and by the authors.

Sphingomyelin values were calculated from the weights (15 to 85 mg.) of dried sphingomyelin reineckate by multiplying by the factor 0.788 (12).

*Determination of Radioactivity of Sphingomyelin Reineckate*—The sphingomyelin reineckate was not sufficiently soluble in

methanol-acetone to be dissolved in the 2 cc. volume necessary for our counting procedure (1). For this reason the sphingomyelin reineckate samples were ashed with fuming nitric acid before the radioactivity was determined. Since the ashing with fuming nitric acid does not completely liberate phosphate from the sphingomyelin compound, the material after counting was heated with concentrated sulfuric acid before the phosphorus analyses could be made.

#### DISCUSSION

*Choice of Solvent for Extraction of Sphingomyelin*—Since it was not known whether alcohol-ether alone as used by Bloor (2) would extract sphingomyelin completely from tissues, it seemed advisable to follow such extraction by treatment with chloroform-methanol (14). Comparative amounts of sphingomyelin obtained by extraction of the wet tumor tissue with alcohol-ether with and without subsequent use of chloroform-methanol are presented in Table I.

Thus with the additional use of chloroform-methanol larger amounts of sphingomyelin are extracted than with alcohol-ether alone. From the small number of analyses it appears to be immaterial whether the residue obtained from such tissue extraction is extracted with petroleum ether or with chloroform.

*Sphingomyelin Content of Carcinosa Sarcoma 256 Tissue*—The sphingomyelin content of whole tumor tissue and that of peripheral and central tumor tissue is given in Table II. These results indicate that sphingomyelin makes up approximately one-quarter of the total phospholipid content of the peripheral tissue of this tumor (6) and considerably more than that of the central tissue. The difference in sphingomyelin content between peripheral and central tissue is not significant and is not accounted for by the difference in water content of these two portions of the tumor (6); in fact, the higher water content of the central tissue would tend to increase this difference when expressed on a dry weight basis. It is interesting to note that the sphingomyelin content of this tumor (dry weight) is higher than that of any normal tissue except brain according to the figures given by Thannhauser *et al.* (13).

*Phosphorus Content of Sphingomyelin*—Evidence has been ad-

vanced (14) to indicate that sphingomyelin may exist in an ester form. This esterified sphingomyelin (palmitylignoceryl sphingo-

TABLE I  
*Sphingomyelin Obtained by Extraction of Wet Tumor Tissue*

Solvent used for extraction of		Per cent sphingo- myelin in tumor (moist weight)
Tumor tissue	Residue	
Alcohol-ether followed by ether	Petroleum ether	0.30
		0.29
		0.24
		0.32
		0.18
		0.23
		0.23
		0.27
0.33		
Average.....		0.27
Alcohol-ether followed by ether then chloroform- methanol	Petroleum ether	0.35
		0.35
		0.43
		0.39
	Chloroform	0.41
		0.34
		0.36
Average.....		0.38

TABLE II  
*Sphingomyelin Content of Tumor Tissue*

Tissue	No. of tumors	Sphingomyelin, average per cent moist weight
Whole.....	25	0.32 $\pm$ 0.04*
Peripheral.....	23	0.30 $\pm$ 0.05*
Central.....	21	0.38 $\pm$ 0.06*

\* Standard deviation.

myelin) would have a theoretical phosphorus content of 2.89 per cent, while that of the unesterified sphingomyelin is 3.72 per cent. Thannhauser and Reichel (14) point out "the possibility that the

lipid exists solely in the esterified form in nature, and that partial autolysis of the ester takes place in the tissue during the process of isolation." The data presented in Table III do not support this idea. The fact that in twenty out of twenty-three analyses a lower phosphorus content is found in the sphingomyelin of the central tissue may indicate that here the sphingomyelin is largely in the esterified form while in the outer or more actively growing tissue the additional fatty acid is lacking. This would agree with the finding (6) of much larger amounts of esterified cholesterol in the center than in the periphery of this same tumor.

TABLE III  
*Per Cent Phosphorus in Sphingomyelin from Peripheral and Central Tumor Tissue*

Peripheral tissue	Central tissue	Difference	Peripheral tissue	Central tissue	Difference
3.50	2.96	0.54	3.76	3.34	0.42
3.45	3.61	-0.16	3.68	3.67	0.01
3.79	3.70	0.09	3.89	3.57	0.32
3.58	3.29	0.29	3.43		
3.86	2.96	0.90	3.20	3.01	0.19
3.87	3.55	0.32	3.23		
3.54	3.59	-0.05	3.37	3.24	0.13
3.82	3.23	0.59	3.69	3.24	0.45
3.57	3.36	0.21	3.66	3.14	0.52
3.77	3.50	0.27	3.12	3.11	0.01
3.23	3.53	-0.30	3.66	3.05	0.61
3.54	3.30	0.24	3.45	3.43	0.02
3.58	3.15	0.43	3.80	3.56	0.24

If sphingomyelin can exist in tissues in both an esterified and an unesterified form, the use of Thannhauser's factor of 0.788 for the calculation of sphingomyelin reineckate as sphingomyelin may not be justified. This factor, obtained empirically by Thannhauser, corresponds closely to the theoretical factor (0.789) for the *unesterified* compound. The theoretical factor for the *esterified* form is 0.828. Consequently the correct factor for use would lie between 0.788 and 0.828, depending on the relative amounts of the two forms of sphingomyelin present in the tissue.

It can be seen then that the values given above for sphingomyelin, calculated from the factor 0.788, must represent minimum

amounts of this lipid in the tissue; similarly the values for the phosphorus content of sphingomyelin given in Table III, having been calculated from these minimum amounts of sphingomyelin, must represent maximum percentages.

The wide range in values for percentage of phosphorus in the sphingomyelin of the periphery as well as in the values for the central tissue is undoubtedly due to the fact that an absolute separation of these two kinds of tissue cannot be obtained.

*Rate of Turnover of Sphingomyelin. Radioactive Phosphorus by Stomach Tube (Series I)*—In Fig. 1 is shown the percentage of

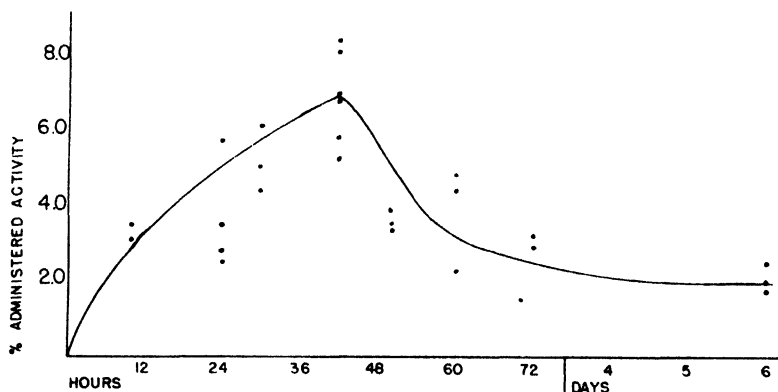


FIG. 1. The percentage of the administered activity per gm. of tumor sphingomyelin at various times after *oral* administration of disodium hydrogen phosphate containing radioactive phosphorus to tumor-bearing rats (Series I). The curve has been placed by inspection.

administered activity found as tumor sphingomyelin at various times from 10 hours to 6 days after administration of the radioactive phosphate solution by stomach tube to tumor-bearing rats. Comparison of these results with those already published for the lecithin and cephalin fractions of this tumor (8) shows that a close similarity exists in the uptake of radioactive phosphorus by these three lipids. The amount of phosphorus replaced in sphingomyelin in a given time is comparable to that replaced in the other two lipids. Also, the time of maximum activity is approximately the same, sphingomyelin being somewhat more like cephalin than lecithin. Similarly, Chargaff *et al.* (3) have reported a cephalin-

like turnover for brain sphingomyelin. While sphingomyelin, as Hunter has shown (9), is responsible for but a small part of the phospholipid phosphorus metabolism in the liver, the sphingomyelin of this tumor plays a large part in its phospholipid metabolism.

*Radioactive Phosphorus Subcutaneously (Series II)*—When radioactive phosphate solution is given by stomach tube, the differing rates of absorption for each animal become an additional variable. In order to rule out this variable, the phosphate solution was administered *subcutaneously* to the animals in Series II. In Table

TABLE IV

*Per Cent of Administered Activity per Gm. of Sphingomyelin from Peripheral and Central Tumor Tissue at Various Times after Administration of Radioactive Phosphorus*

Time after administration	Peripheral tissue	Central tissue	Time after administration	Peripheral tissue	Central tissue
<i>hrs.</i>			<i>hrs.</i>		
6	4.7	2.4	34	7.3	5.2
6	4.6	3.6	42	7.5	4.6
6	3.6	3.6	42	9.2	5.3
12	11.3	2.9	50	5.8	3.9
12	5.5	3.1	50	6.9	4.9
18	5.7	2.0	50	7.1	4.6
18	6.0	3.1	60	7.1	5.8
24	6.5	4.1	60	7.5	4.7
24	7.6	4.3	72	7.2	4.4
24	8.2	4.8	72	7.9	7.3
34	6.8	3.7	96	7.4	6.1
34	5.6	4.2			

IV are shown the values obtained on sphingomyelin isolated from the periphery and from the center of twenty-four tumors. Again the fact that an absolute separation of peripheral and central tissue could not be obtained accounts for the range in values on each tissue. It will be noted that in each case the value for the center is lower than that for the corresponding periphery. This difference can be accounted for by the better blood supply to the periphery and the fact that this tissue is actively growing.

The values for activities of peripheral and central sphingomyelin of each tumor (Table IV) have been combined and, together with data from five additional tumors, are presented in Fig. 2. Com-



parison of Fig. 1 and Fig. 2 reveals that, in general, the values in Fig. 2 are higher than those at corresponding times in Fig. 1. Moreover when the radioactive phosphorus is administered subcutaneously, there is no pronounced peak in activity; in fact, the values at 42 hours (the time when activity is greatest after administration by stomach tube) are lower in Fig. 2 than in Fig. 1. These discrepancies must be due to a different distribution of the isotope when administered by the two methods. On the one hand, when administered orally, the radioactive phosphorus is transported first to the liver via the portal circulation from the

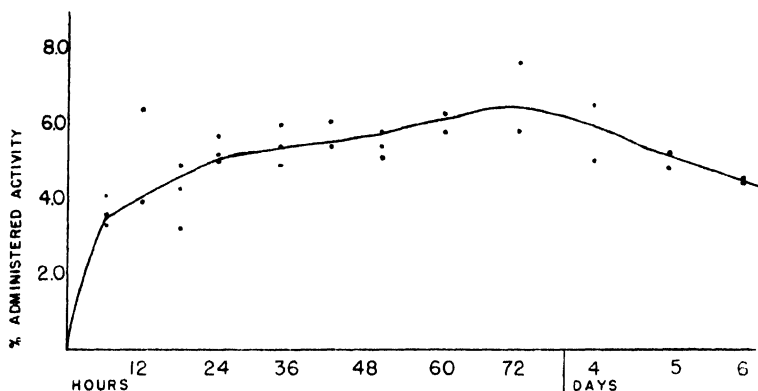


FIG. 2. The percentage of the administered activity per gm. of tumor sphingomyelin at various times after *subcutaneous* administration of disodium hydrogen phosphate containing radioactive phosphorus to tumor-bearing rats (Series II). The curve has been placed by inspection.

intestine; on the other hand, when administered subcutaneously, the radioactive isotope is distributed over the body by the systemic blood.

#### SUMMARY

1. Sphingomyelin has been found to occur in rat Carcinosarcoma 256 to the extent of about 0.3 per cent of the wet weight; there is slightly more of this lipid in the less actively growing center of the tumor than in the actively growing periphery.

2. Sphingomyelin isolated from the periphery of the tumor contains a higher percentage of phosphorus than that isolated from

the center; this fact may indicate that this lipid occurs in the esterified form in the center of the tumor.

3. The incorporation of radioactive phosphorus into tumor sphingomyelin is greatest at 42 hours after administration of the isotope by stomach tube, while the activity after subcutaneous administration exhibits no such maximum. The activities are in general higher after subcutaneous than after oral administration.

4. Tumor sphingomyelin plays as important a rôle in phospholipid phosphorus metabolism as do lecithin and cephalin.

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# **DISTRIBUTION AND EXCRETION STUDIES IN THE RAT AFTER A SINGLE SUBTOXIC SUBCUTANEOUS INJECTION OF SODIUM SELENATE CONTAINING RADIOSELENIUM\***

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Previous investigators (1-4) have demonstrated a wide but varied distribution of selenium in animal tissues by feeding and injection of single and multiple, minimal, subtoxic, and toxic doses of both inorganic and organic forms. Their work has been extended by the utilization of the radioactive isotope of selenium as a tagged atom. With the use of radioselenium which has advantages of a half life of 50 days with a specific  $\gamma$ -ray emission of 0.5 and 0.21 m.e.v. (5) micro selenium determinations have been made with far greater precision than would have been possible by any known chemical analytical procedure. The extreme sensitivity of the method has made possible not only quantitative detection of traces of the metal but a study of time-distribution differences of exceedingly small amounts. This has enabled the investigator to study the mechanism of excretion accurately, as well as the exchange of selenium from one tissue to another. By means of this anomalous method, it was the plan of the experiments presented here to make a thorough time-distribution and excretion study in the rat after a single injection of sodium selenate containing radioselenium.

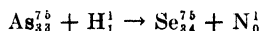
## *Method*

Sodium selenate containing radioselenium was injected subcutaneously into twenty-five young, adult, male, stock, albino

\* This study was supported in part by a grant-in-aid from the Rockefeller Foundation.

rats, thirteen of which at the time of selenium injection bore small implanted, transplanted, 3 week-old tumors of Carcinosarcoma 256 in the groin. Each rat received a single, subcutaneous dose of 0.28 to 0.72 mg. of selenium as sodium selenate. The animals were placed in metabolism cages designed for separate collection of urine and feces (6). Food (Purina Chow) and water were administered *ad libitum*. The rats were killed by decapitation at various time intervals up to 96 hours, and the radioactivity of the tissue determined. The red blood cells and plasma were separated in the usual manner by centrifugation, and the cells were washed twice with isotonic saline and the washings added to the plasma fractions.

Radioselenium was produced in the Department of Physics of The University of Rochester by Dr. Van Voorhis and his group by bombarding arsenic-iron alloy in the cyclotron with high speed protons. The equation for the formation of radioselenium is as follows:



Radioselenium decays through K electron capture, emitting two quantum  $\gamma$ -rays with the formation of stable  $\text{As}_{33}^{75}$  (5).

It was necessary to separate radioselenium from iron, arsenic, and various substances resulting from the bombardment of the arsenic alloy. The radioactive sample was placed in 25 cc. of chilled, concentrated sulfuric acid to which were added 100 cc. of 5 per cent bromine and 48 per cent hydrobromic acid solution which contained a known amount of non-radioselenium to act as a carrier for the radioselenium. The mixture was then distilled in an all-glass distilling apparatus, and the selenium recovered from the distillate by reducing the selenium bromide with sulfur dioxide. This mixture of radioselenium and a known amount of non-radioselenium afforded the starting material for the synthesis of sodium selenate. A modification of Gilbertson and King's method for the synthesis of selenic acid was used (7). Selenic acid was obtained as a very viscous, colorless liquid which was diluted with water and titrated against standard alkali with phenolphthalein as an indicator to form sodium selenate. The titration of four samples showed that 98.8, 104.5, 95, and 96 per cent of the selenium was converted to selenic acid. Qualitative tests show that selenic acid was formed.

The wet ashing technique with concentrated nitric acid followed with 30 per cent hydrogen peroxide was used to prepare the tissues for radioactive measurements. These were made on a Geiger-Müller counter according to the method described by Bale, Haven, and LeFevre (8). A recovery study was made. A known amount of radioselenium was added as sodium selenate to various tissues which were wet ashed, and the radioactivity determined. The re-

TABLE I  
*Recovery of Selenium Added to Tissues*

The *average net count* represents the average count per minute (minus background) obtained from three 5 minute determinations which were made on a Geiger-Müller counter of a scale-of-four. Values for the *original standard* were obtained from an aliquot of sodium selenate containing radioselenium. For a *control*, an equivalent amount of radioselenium was added to the amounts of  $\text{HNO}_3$  and  $\text{H}_2\text{O}_2$  that were used for ashing tissues and then carried through all the experimental steps. An equivalent amount of radioselenium was then added to various tissues which were wet ashed and the radioactivity determined.

Tissue	Average net count	Average deviation
Original standard .....	15.1	0.73
Control (with all steps through the reagents)...	14.7	1.40
4 gm. normal liver + same amount standard...	16.2	1.23
4 " " " + " " " *	14.9	2.00
2 " bone + same amount standard .....	15.7	0.40
1.5 gm. kidney + same amount standard .....	17.0	0.57
5 cc. whole blood + same amount standard .....	16.6	0.63

\* 0.5 gm. of  $\text{HgO}$  was added to investigate whether  $\text{HgO}$  prevented the loss of selenium in the ashing method used.  $\text{HgO}$  does not increase the recovery of selenium.

sults in Table I show that added selenium can be quantitatively recovered within the limits of the counting technique used.

#### DISCUSSION

In Figs. 1 to 4, radioactivity for the various tissues is expressed as the per cent of the dose per gm. of tissue; for whole blood, cells, and plasma as per cent of the dose per cc.; and for urine and feces as per cent of the dose. The *whole blood* (Fig. 1) reaches a maximum concentration around the first quarter hour, followed by a

rapid decrease in concentration for the first 24 hours, and then a slower decrease over the second and third 24 hour periods. When the percentage of the dose for whole blood<sup>1</sup> was calculated for the total blood of the animal, it was found that 6.4 per cent of the original dose was in the blood at the first quarter hour, whereas at the  $\frac{1}{2}$ , 2, 12, and 24 hour periods the percentages were 5.6, 4.7, 2.6,

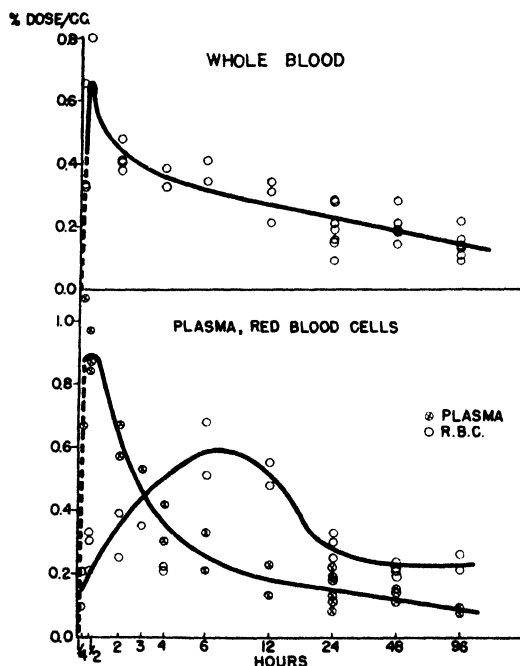


FIG. 1. Time-distribution of selenium in whole blood, plasma, and red blood cells after injection of sodium selenate. Each circle represents an individual experiment.

and 1.7 respectively. Examination of Fig. 1 reveals that  $\frac{1}{2}$  hour after injection, the ratio of selenium in *plasma* and *red blood cells* is approximately 6.2:1.0, and at the 2nd hour is 3.2:1.0. At some time between the 2nd and 6th hours, the ratio is 1:1, while at the 6, 12, 24, and 48 hour periods the ratios are 1:2, 1:2.8,

<sup>1</sup> Total blood volumes were calculated from body weights by means of the conversion tables given by Donaldson (9).

1:1.7, and 1:1.7 respectively. It is apparent therefore that selenium first appears in the blood in greater amounts in the plasma than in the red blood cells, and is removed from the plasma faster than from the red blood cells. It would appear from the data (Fig. 1) that the rate of deposition of selenium in red blood cells was more rapid than the rate of elimination, and that a certain amount of fixation of selenium occurs. Further studies on

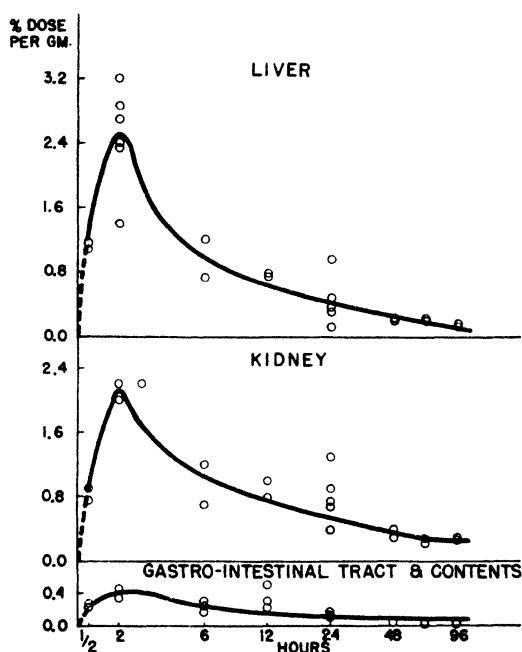


FIG. 2. Time-distribution of selenium in various tissues after injection of sodium selenate.

the fractions of plasma and red blood cells are being carried out in order to identify the selenium-carrying portions.

The concentration of selenium rises to a higher level in the *liver* than that found in any other organ under the conditions of the experiment. The results in Fig. 2 expressed as percentages of the dose per gm. of liver show that the maximum concentration of selenium in the liver occurs at the 2nd hour shortly after the maximum in the whole blood. There was a faster rate of elimination



during the first 24 hours than at any time during the second, third, and fourth 24 hours. Calculated on the basis of per cent of the original dose per organ, 6.1, 18.9, 5, and 2.5 per cent were found in the liver at  $\frac{1}{2}$ , 2, 12, and 24 hours, respectively. In the *kidney*, as in the liver, a maximum concentration was reached at the 2nd hour, followed by a more rapid decrease during the remainder of the first 24 hours than during the following 24 hour periods. At the  $\frac{1}{2}$ , 2, 12, and 24 hour periods, the percentage of dose per organ was 1.2, 4.2, 1.3, and 0.9, respectively. Peak concentrations in

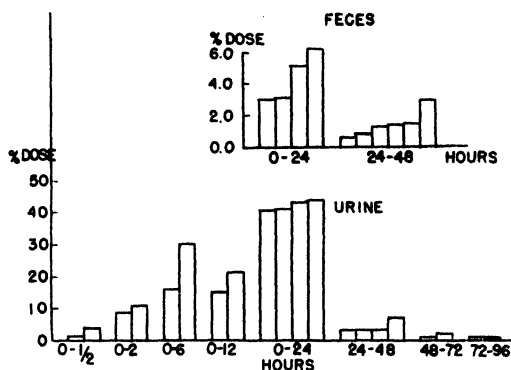


FIG. 3. Excretion of selenium in urine and feces after injection of sodium selenate. Each bar represents an individual experiment, and the urine values include bladder urine.

the liver, kidney, and gastrointestinal tract are the highest of all the tissues examined, and it is reasonable to believe that these tissues are more vulnerable to injury than any other tissues studied. The smaller concentrations, as found in the lung, heart, etc., apparently cause little damage there. This is borne out by histological observations made by us<sup>2</sup> which are essentially a confirmation of the findings of several investigators as reviewed by Stenn (10). The kidney secretes selenium in the urine at a faster rate during the first 24 hours than during the following two 24 hour periods (Fig. 3), for during the first 24 hours, 40 per cent of the original dose appeared in the urine, while during the second, third, and fourth 24 hours, 3.4, 1.5, and 1.1 per cent, respectively, of the origi-

<sup>2</sup> Warren, S. L., and McConnell, K. P., unpublished data.

nal dose was excreted. There was no apparent relationship between the concentration in the kidney and the excretion of selenium in the urine.

Urine from an animal that had been injected subcutaneously with  $\text{Na}_2\text{SeO}_4$  was collected under mineral oil. The radioactivity of an aliquot of collected urine was compared with that of three

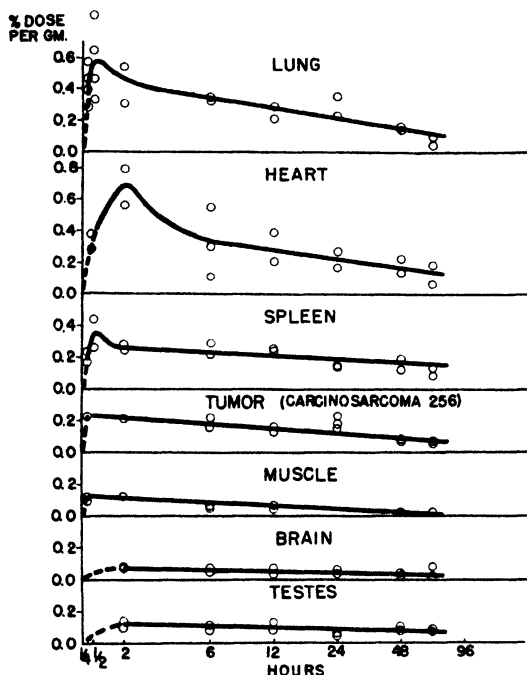


FIG. 4. Time-distribution of selenium in various tissues, including the rat tumor, Carcinosarcoma 256, after injection of sodium selenate.

aliquots that had been evaporated to dryness on a steam bath. The aliquot not evaporated had a count of 83.5, while the three equal aliquots that had been evaporated to dryness had counts of 80.0, 81.0, and 77.0, respectively. It follows that selenium in the urine was non-volatile at steam bath temperature. Three portions of urine collected as indicated above were treated as follows: one was made acid with sulfuric acid, one was made basic with  $\text{NaOH}$ , and the third was untreated (pH 5.8). The three frac-

tions were extracted repeatedly with ether. The ether fractions were washed with water and subsequently dried with  $(\text{NH}_4)_2\text{SO}_4$ . The ether fractions were then evaporated to dryness and the residual material taken up in concentrated  $\text{HNO}_3$  and the radioactivity determined. The results in Table II show that selenium was not in an ether-soluble form whether the urine was acidic, basic, or normal, indicating that selenium was excreted during the first 24 hours in an inorganic form.<sup>3</sup>

As shown in Fig. 2, the per cent dose per gm. of *gastrointestinal tract* (both tissue and contents) increases up to the 2nd hour, after which a steady decrease takes place until the 48th hour, after which only traces are present up to the 96th hour. On the basis of percentage dose per whole organ at  $\frac{1}{2}$ , 2, 12, and 24 hours, the

TABLE II  
*Rat Urine Studies after Injection of Selenium*

Urine sample	Counts (average of three)*	
	Ether fraction	HOH fraction
Untreated, extracted.....	0.0	132.5
Acid.....	0.0	127.5
Basic.....	0.0	131.5
Whole, not extracted.....		133.5

\* Made on a Geiger-Müller counter.

percentages are 5.6, 10.3, 5.7, and 1.5, respectively. Excretion of selenium by way of the gastrointestinal tract was far less than by way of the kidney. The per cent dose of selenium in the *feces* (Fig. 3) was greater during the first 24 hours than during the following 24 hour period. 3 to 6 per cent of the dose appeared in the feces during the first 24 hours, while only 1 to 3 per cent was found during the second 24 hours. The *lung* reaches a peak concentration soon after injection (Fig. 4), namely at  $\frac{1}{2}$  hour, after which a steady decrease appears for the ensuing 72 hours. At

<sup>3</sup> Since this paper was prepared for publication, Westfall and Smith (11) have reported in urinary selenium studies in rabbits that 85 per cent of the urinary selenium excreted in 24 hours was in the  $\text{BaSO}_4$ -ethyl alcohol fraction, which is further evidence that selenium when administered as  $\text{Na}_2\text{SeO}_4$  is excreted in the urine as inorganic selenate.

$\frac{1}{2}$  hour the lung has a value of 0.57 per cent, calculated on the basis of per cent dose per organ.

The peak concentration appeared in the *spleen* at  $\frac{1}{2}$  hour and in the *heart* at 2 hours. The rate of deposition of selenium for heart and spleen as well as for other viscera was much more rapid than the rate of elimination. Expressed as per cent dose per organ at  $\frac{1}{2}$  hour, the concentration in the spleen was 0.14 per cent, while it was 0.53 per cent for the heart at 2 hours. *Tumor tissue* (from Carcinosarcoma 256) absorbs selenium in small but significant amounts, 1.2 per cent of the dose being found in the entire tumor at  $\frac{1}{4}$  hour.

The total *body musculature* was calculated from body weights by means of conversion tables by Donaldson (9). Counts for muscle were obtained on the gastrocnemius muscle. The per cent dose per total body musculature at  $\frac{1}{4}$ , 2, 6, 12, and 48 hours was found to be 8.2, 10.0, 2.6, 2.3, and 1.2 per cent respectively. The *testes* and *brain* at various time intervals from 2 to 72 hours had at the most only traces of selenium. The *fur* was removed by means of clippers from the *skin* of three animals that had previously been injected subcutaneously with  $\text{Na}_2\text{SeO}_4$ . 24 hours after injection no radioselenium could be detected in either the skin or fur.<sup>4</sup>

The *teeth* (incisors) and *long bones* for the 24 hour period were crushed, dissolved separately in 6 N HCl, and made up to volume for radioactive determinations by Doctor Volker of the Dental Research group. Results show that radioselenium was not present in either tooth or bone substance at the 24 hour period.

The possibility that the concentration of selenium in tissues with trace amounts of selenium (brain, testes, etc.) may be due in part to the presence of selenized blood has, of course, not been overlooked. However, in such tissues as liver, kidney, muscle, and gastrointestinal tract in which the concentration of selenium far exceeds that which might be accounted for by the presence of selenized blood, the greater part of the selenium present is unquestionably absorbed selenium. Hence, after subcutaneous injection of subtoxic amounts of sodium selenate, selenium enters the blood stream, from which rapid selective absorption by various tissues

<sup>4</sup> The method of Robinson *et al.* (12) for the isolation of selenium from biological material was used. 10 mg. of added non-radioselenium acted as a carrier for radioselenium.

takes place followed by a slow elimination into the blood stream with subsequent excretion chiefly by way of the kidney.

#### SUMMARY

With radioselenium as a tagged atom, it has been possible to determine the distribution and excretion of a single, subtoxic, subcutaneous injection of sodium selenate at various periods up to 96 hours, and to compare quantitatively peak concentrations of selenium in the various tissues including tumor Carcinosarcoma 256 of the white rat.

A wide but varied distribution of selenium was found in the tissues examined. Only a small percentage of the original dose was found in any of the tissues at any one time, the highest average concentration, 19 per cent, being found in the liver. The greatest concentration appeared in liver, muscle (total), gastrointestinal tract, and blood; lesser amounts in lung, spleen, heart, and tumor; and traces in brain and testes. None was found after 24 hours in skin, fur, teeth, or long bones. The maximum concentration for individual tissues appeared in blood and lung before appearing in liver, kidney, and gastrointestinal tract. Small amounts of selenium of the magnitude of 1 per cent were deposited in rat tumor, Carcinosarcoma 256.

A rapid rate of deposition of small amounts of selenium within a few hours after injection followed by a prolonged, less rapid rate of elimination was characteristic of most tissues.

Within the first half hour (the time at which the greatest concentration of selenium was found in blood), it appeared in greater concentration in the plasma than in the red blood cells. Following this, the concentration in blood was diminished, owing to the fact that the concentration decreases more rapidly in plasma than it increases in red blood cells. After the 3rd hour, the concentration in the red blood cells exceeded that found in the plasma.

Selenium was excreted chiefly by way of the kidney in a non-volatile, ether-insoluble form, and to a lesser extent by way of the gastrointestinal tract. 41 to 43 per cent of the original dose appeared in the urine, while only 3 to 6 per cent was found in the feces during the first 24 hour period. Much smaller amounts of selenium appeared in both urine and feces during the second and ensuing two 24 hour periods.

The author wishes to express his appreciation to Dr. William F. Bale who was responsible for stimulating interest in metabolic experimentation with radioselenium. He would also like to thank Dr. Stafford L. Warren, Dr. Robert R. Sealock, and Dr. James H. Sterner for their helpful advice and criticism throughout this investigation.

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## CARBON DIOXIDE UTILIZATION BY PIGEON LIVER\*

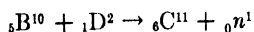
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Direct experimental evidence that carbon dioxide is used in the formation of  $\alpha$ -ketoglutaric acid from pyruvic acid by a suspension of minced pigeon liver has been previously outlined (1, 2). The present report comprises a detailed description of this reaction and of the process of carbon dioxide assimilation by pigeon liver. The principal facts found are: (1)  $\alpha$ -Ketoglutaric acid synthesized from pyruvic acid in a radioactive bicarbonate medium contains radioactive carbon. (2) On the average about 5 to 10 per cent of the total radioactivity of the system can be accounted for by the isolated  $\alpha$ -ketoglutaric acid. (3) All of the radioactive carbon in the  $\alpha$ -ketoglutaric acid is present in the carboxyl group  $\alpha$  to the ketonic oxygen. (4) The addition of citric acid to the system during the synthesis of  $\alpha$ -ketoglutarate from pyruvate causes no change in the ratio of activity per mg. of carbon of the  $\alpha$ -ketoglutarate to that of the medium. (5) Only about 25 per cent of the assimilated carbon dioxide can be accounted for as  $\alpha$ -ketoglutaric acid. (6) A part of this residual (non- $\alpha$ -ketoglutarate) radioactivity can be released as carbon dioxide by treatment with ninhydrin and with chloramine-T. (7) An assimilation of  $\text{CO}_2$  similar to that in liver does not occur in the formation of  $\alpha$ -ketoglutarate by minced pigeon muscle.

*Preparation of  $\text{C}^{11}\text{O}_2$* —Radioactive  $\text{C}^{11}$  was prepared by the bombardment of amorphous boron with 8.5 m.e.v. deuterons in the Chicago cyclotron, the reaction being,



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After bombardment, the boron was mixed with twice its weight of powdered CuO and heated at 900° in a stream of oxygen in a quartz combustion tube. The C<sup>11</sup>O<sub>2</sub> was frozen out over 0.1 ml. of N NaOH contained in a glass trap immersed in liquid nitrogen. The small quantity of alkaline solution containing the radioactive carbon was transferred quantitatively to the experimental vessel which contained from 50 to 100 ml. of Krebs' saline-bicarbonate medium, previously equilibrated with 95 per cent oxygen, 5 per cent CO<sub>2</sub>. The introduction of the small quantity of carbonate containing the radioactive carbon caused no appreciable change in the pH or ionic concentration of the medium. The time required for these operations, from the removal of the target from the cyclotron to the introduction of the radioactive carbonate into the experimental solution, was about 15 minutes. More than 95 per cent of the radioactivity of the target material could be recovered as C<sup>11</sup>O<sub>2</sub> and the addition of C<sup>12</sup> as a carrier was not necessary. The activity was sufficient to permit a working period of 3.5 to 4 hours, despite the short half life of the radioactive carbon and dilution of the carbon dioxide in the course of the experiment.

*Measurement of Radioactivity*—Activities (expressed as divisions per second) were measured with a Lauritsen electroscope. To make the readings comparable and to correct for self-absorption all measurements were made in solution and under identical geometrical conditions. In every case the unknown was dissolved in 0.5 ml. of either water or alkali. Duplicate samples could be read with an error of less than 2 per cent. In Tables I to IV, values for radioactivity have been corrected for decay and are comparable within a given experiment.

*Distribution of Radioactive Carbon Dioxide in Bicarbonate Medium*—The method of experimentation consisted in shaking the minced tissue in Krebs' Ca-free saline medium, pH 7.4, in an atmosphere of 95 per cent oxygen, 5 per cent CO<sub>2</sub>. It was desirable to know, therefore, how rapidly the radioactivity is distributed between the gas and liquid phases of the experimental system after its addition as radioactive carbonate. Experiments to answer this question were devised as follows: 50 ml. of the bicarbonate buffer, pH 7.4, to which has been added 0.3 ml. of radioactive carbonate were shaken in an atmosphere of 95 per cent oxygen, 5 per cent CO<sub>2</sub> in an experimental vessel having a volume of 203 ml. At

intervals during the shaking 0.5 cc. portions of the liquid phase were withdrawn by means of a calibrated syringe through the rubber stopper which closed the shaking vessel, immediately mixed with a known volume of strong alkali by way of a 2-way stop-cock inserted in the syringe barrel, and an aliquot of this solution was used for radioactivity measurements. The same procedure was adopted in similar experiments in which 7.6 gm. of freshly minced pigeon breast muscle were added. Fig. 1 depicts two such ex-

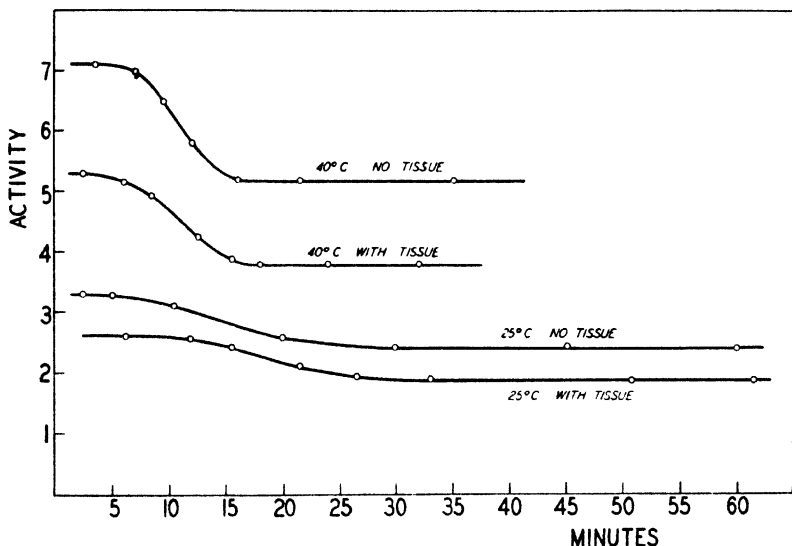


FIG. 1. Exchange rate of radioactive  $\text{CO}_2$  between liquid and gas phases, in bicarbonate medium.

periments carried out at 25° and 40° respectively. At 25° complete equilibrium is attained 23 minutes after the addition of the radioactive carbonate, while at 40° equilibrium is reached in 14 minutes. It will be noted that at neither temperature does the presence of tissue markedly influence the rate of attainment of equilibrium.

*Utilization of  $\text{CO}_2$  in  $\alpha$ -Ketoglutarate Synthesis*—The experimental procedure was as follows: 100 ml. of Ca-free Krebs' bicarbonate-saline were shaken at 40° for 10 minutes while a vigorous stream of 95 per cent oxygen, 5 per cent  $\text{CO}_2$  was bubbled through

the solution. The radioactive carbonate (0.3 ml.) was added together with 1.7 ml. of 0.1 M malonate (3) and the experimental vessel closed and shaken for 15 minutes. 7.6 gm. of freshly minced pigeon liver were next added together with 10 ml. of 0.2 M pyruvate. The vessel was closed and shaken for a few minutes and 0.5 ml. of the suspension was withdrawn for a determination of the total original activity of the solution. The reaction was then let proceed while the closed vessel was shaken vigorously for 40 minutes. At the end of this time practically all of the pyruvate had been utilized. Three 0.5 cc. samples were withdrawn from the solution. One of these was mixed immediately with strong alkali and measured for total radioactivity. The second sample was pipetted immediately into 3 cc. of 2 N NaOH contained in Warburg vessels and, after acidification, the quantity of CO<sub>2</sub> determined manometrically. The third aliquot was pipetted into an alkaline solution, acidified, and the liberated CO<sub>2</sub> swept over by a stream of nitrogen into an absorbing tube containing 5 ml. of 20 per cent KOH. An aliquot of this was removed to determine the radioactivity of the liberated CO<sub>2</sub>. These determinations permit a measure of the amount and radioactivity of the total CO<sub>2</sub> of the medium at the end of the experimental period.

After removal of these samples, the reaction mixture was deproteinized with 25 ml. of 10 per cent metaphosphoric acid. The volume of the filtrate was measured and two 5 cc. aliquots were removed for simultaneous determinations of  $\alpha$ -ketoglutarate and succinate as described by Krebs and Johnson (4). 25 mg. of  $\alpha$ -ketoglutaric acid were added to the remaining filtrate to act as a carrier and the solution mixed in centrifuge cups with an equal volume of a saturated solution of 2,4-dinitrophenylhydrazine in 2 per cent HCl. The hydrazone separated immediately and was collected by centrifuging. The precipitate was dissolved in boiling 60 per cent alcohol and the solution filtered into centrifuge tubes contained in an ice bath. The hydrazone crystallized quickly and was collected by centrifuging and dried *in vacuo* at 100°. The precipitate was weighed and dissolved in 0.5 ml. of 2 N NaOH for measurement of its radioactivity. The hydrazone melted at 223° and gave no depression of the melting point when mixed with a known sample.

On recrystallization the hydrazone gave material with a con-

stant rate of decay per mg. (Experiment 3, Table I), indicating the identity of the radioactivity with the  $\alpha$ -ketoglutarate dinitrophenylhydrazone.

Control experiments in the absence of tissue were carried out in which amounts of pyruvic acid and  $\alpha$ -ketoglutaric acid, comparable to those used in the tissue experiments, were shaken for 40 minutes at 40° in a radioactive bicarbonate buffer, pH 7.4. The dinitrophenylhydrazones of the keto acids were isolated and found to be completely devoid of radioactivity.

It is theoretically possible to ascertain the number of moles of CO<sub>2</sub> used per mole of  $\alpha$ -ketoglutarate synthesized by a com-

TABLE I

*Synthesis of  $\alpha$ -Ketoglutarate in Radioactive Bicarbonate Medium*

The activities are expressed as divisions per second.

Experiment No.	$\alpha$ -Keto-glutarate synthesized	Activity of $\alpha$ -ketoglutarate		Total activity of medium	Terminal activity of medium per mg. inorganic C	Ratio, $\alpha$ -ketoglutarate activity to original activity of medium	Ratio, medium activity per mg. C to $\alpha$ -ketoglutarate activity per mg. C
		Total	Per mg. C				
	mg.						
1	31.4	0.471	0.036	8.74	0.078	0.054	2.0
2	27.5	4.09	0.362	67.0	1.13	0.061	3.1
3	55.0	3.85	0.171	79.8	1.17	0.048	6.9
			0.161*				
			0.178*				

\* Activities after successive recrystallizations of  $\alpha$ -ketoglutarate dinitrophenylhydrazone.

parison of the radioactivity per mg. of carbon of the  $\alpha$ -ketoglutarate at any given moment with the radioactivity per mg. of carbon of the inorganic carbon of the medium. Under the experimental conditions used, however, any such comparison is complicated by the continuous metabolic production of large amounts of non-radioactive CO<sub>2</sub> during the course of the experiments, the assimilation of CO<sub>2</sub> in reactions other than  $\alpha$ -ketoglutarate synthesis, the length of time required for a uniform distribution of radioactive carbon between liquid and gas phases, and the possible preferential synthetic utilization of non-radioactive carbon dioxide which is produced in the tissue in the immediate vicinity of the synthesizing enzyme systems.

Quantitative data from a series of experiments are shown in Table I. In a typical experiment such as No. 3, the original radioactivity was 88 divisions per second for 60 mg. of CO<sub>2</sub> in the liquid and 14 mg. of CO<sub>2</sub> in the gas phase. During the 40 minutes of the experiment about 80 mg. of metabolic CO<sub>2</sub> were formed (3). Assuming no assimilation of CO<sub>2</sub>, this would reduce the radioactivity of the medium to 2.09 divisions per second per mg. of carbon. Utilization of CO<sub>2</sub> occurs, however, as evidenced by the terminal activity of the medium being 1.17 divisions per second per mg. of carbon. Almost half of the activity originally present as carbonate has been converted into a bound form not liberated by acid. If the average activity of  $\alpha$ -ketoglutaric acid is calculated on the basis of a constant rate of synthesis in a medium having an initial activity of 2.09 divisions per second per mg. of inorganic carbon and a final activity of 1.17 divisions, an average value for the acid of 1.63 divisions per second is obtained. The observed value for Experiment 3 is 0.171. On the basis of this very approximate calculation about 1 carbon atom in 10 of the synthesized  $\alpha$ -ketoglutarate is derived from the medium. The experiments of the next section demonstrate that the number of carbon atoms of the  $\alpha$ -ketoglutarate derived from the medium cannot exceed 1 in 5. Since calculation of the 1 in 10 value is approximate and can indicate only the order of magnitude of the synthesis, it is probable that 1 mole of CO<sub>2</sub> is derived from the medium for every mole of  $\alpha$ -ketoglutarate synthesized.

*Location of Radioactivity in Synthesized  $\alpha$ -Ketoglutaric Acid*<sup>1</sup>—When the radioactive  $\alpha$ -ketoglutarate dinitrophenylhydrazone obtained in the previous experiments is oxidized with potassium permanganate in acid medium at room temperature, a complete loss of radioactivity in the form of CO<sub>2</sub> occurs. In the experiments of Table II the hydrazone was dissolved in 5 cc. of dilute alkali, the solution acidified with 3 cc. of 50 per cent H<sub>2</sub>SO<sub>4</sub>, 10 ml. of saturated KMnO<sub>4</sub> added, and the resulting CO<sub>2</sub> swept over by N<sub>2</sub> into a receiver containing 3 ml. of 5 N NaOH.

<sup>1</sup> A summary of the data in this section was reported (2) at the Symposium on Carbohydrate Metabolism held April 18, 1941, at the meeting of the American Society of Biological Chemists at Chicago. Wood, Werkman, Hemingway, and Nier (5) have independently obtained similar results using the carbon isotope C<sup>13</sup>.

If  $\alpha$ -ketoglutaric acid was synthesized in pigeon liver by the reactions of the citric acid cycle (1), the intermediate formation of the symmetrical citric acid molecule would occur. On oxidation this would yield a mixture of  $\alpha$ -ketoglutaric acid molecules in half of which the radioactive carbon was  $\alpha$  and in the other half  $\gamma$  to the carbonyl group. On oxidation, such a mixture should give succinic acid with a loss of half the original radioactivity as carbon

TABLE II

*Oxidation of Radioactive  $\alpha$ -Ketoglutarate Dinitrophenylhydrazone to Succinic Acid and  $\text{CO}_2$*

The activities are expressed as divisions per second.

Experiment No.	$\alpha$ -Ketoglutarate dinitrophenylhydrazone	Activity	Activity of $\text{CO}_2$ liberated on oxidation
	mg.		
1	55.0	0.23	0.20
2	47.9	0.35	0.33
3	40.0	0.18	0.20

TABLE III

*Effect of Sodium Citrate on Radioactivity of Synthesized  $\alpha$ -Ketoglutaric Acid*

The activities are expressed as divisions per second.

Experiment No.	Citrate added	$\alpha$ -Ketoglutarate synthesized	Activity of $\alpha$ -ketoglutarate per mg. C
	mg.	mg.	
1	0	31.4	0.148
	25	28.8	0.113
2	0	36.9	0.116
	25	39.8	0.116

dioxide. The localization, however, of the entire radioactivity of the  $\alpha$ -ketoglutarate in the carboxyl group adjacent to the carbonyl group as demonstrated in Table II is definite evidence against the suggested formation of this compound from citric acid.

If citrate was an intermediate in the formation of  $\alpha$ -ketoglutaric acid from pyruvate and  $\text{CO}_2$ , the addition of citrate to the synthesizing tissue would give rise, presumably, to  $\alpha$ -ketoglutaric acid of which the radioactivity had been considerably diluted. However, the experiments of Table III show that the addition of 25

mg. of sodium citrate affects neither the yield of  $\alpha$ -ketoglutaric acid nor the ratio of activity per mg. of carbon of the  $\alpha$ -ketoglutarate to that of the medium. These experiments again support the view that citric acid is not an intermediate in the synthesis of  $\alpha$ -ketoglutarate from pyruvic acid and CO<sub>2</sub> by liver under the conditions of these experiments.

*Non- $\alpha$ -Ketoglutaric Radioactivity and Its Nature*—In Table IV data are listed which would indicate that the total amount of CO<sub>2</sub> assimilated is much greater than can be accounted for by the amount of  $\alpha$ -ketoglutaric acid formed. In these experiments the deproteinized reaction mixture was divided into two parts. To one 25 mg. of  $\alpha$ -ketoglutaric acid were added and the dinitro-

TABLE IV

*Effect of Ninhydrin and Chloramine-T on Non- $\alpha$ -Ketoglutarate Radioactivity*

The activities are expressed as divisions per second.

Experiment No.	$\alpha$ -Keto-glutarate synthesized	Total activity of $\alpha$ -keto-glutarate	Total activity of medium after CO <sub>2</sub> removal	Activity of CO <sub>2</sub> released by ninhydrin	Activity of CO <sub>2</sub> released by chloramine-T
	mg.				
1	35.0	5.18	31.8	7.8	
2	39.0	6.2	31.2		10.39
3	53.0	1.2	4.2		0.78
4	31.6	0.22	0.929	0.209	

phenylhydrazone isolated as previously described. A vigorous stream of CO<sub>2</sub> was bubbled through the other portion which was strongly acid. Samples were withdrawn from this solution at intervals until there was no further loss of activity. Under these circumstances it was found that the residual radioactivity was 3 to 4 times greater than that of the synthesized  $\alpha$ -ketoglutaric acid.

Some indication as to the nature of this activity could be derived from the fact that on treatment with ninhydrin at boiling temperature (6) loss of radioactive CO<sub>2</sub> could be demonstrated. A similar reaction occurred with chloramine-T at 40° under the conditions described by Cohen (7). Here the radioactivity of the liberated CO<sub>2</sub> was about one-fourth of the total residual activity. Control experiments indicated that  $\alpha$ -ketoglutaric acid was not decarboxylated by ninhydrin or chloramine-T under the

conditions of these tests. It is probable, therefore, that the activity released by these agents is present as a carboxyl group of amino acids. In view of the existence of the transaminating enzymes in the liver such an amino acid as glutamic acid could be directly formed from the synthesized  $\alpha$ -ketoglutaric acid and would liberate  $\text{CO}_2$  under these conditions. Further examination of the compounds involved is being made.

*Synthesis of  $\alpha$ -Ketoglutaric Acid in Muscle*—In the preceding sections it has been assumed that the appearance of radioactivity in  $\alpha$ -ketoglutaric acid indicates a direct synthetic utilization of carbon dioxide in the formation of this substance. It is possible, however, that the radioactivity could result by a simple process of exchange between an intermediate in the reaction and carbon dioxide of the medium. Current theories of the mechanism of formation of  $\alpha$ -ketoglutarate in pigeon liver postulate an initial condensation of pyruvic acid and  $\text{CO}_2$  to form oxalacetic acid (1, 5, 8). Oxalacetic acid is known to break down in the presence of tissue to  $\text{CO}_2$  and pyruvic acid and it might be argued that any reaction involving this compound would yield a radioactive end-product if the reaction medium contained radioactive carbon dioxide. Experiments to control this possibility are difficult to conceive but a comparison of the radioactivity of the  $\alpha$ -ketoglutaric acid synthesized in muscle with that formed in liver offers some evidence on this point. In muscle  $\alpha$ -ketoglutaric acid is formed by the oxidation of citric acid resulting from a condensation of oxalacetic acid and pyruvic acid (9). A formation of oxalacetic acid from pyruvate and  $\text{CO}_2$  in this tissue does not occur, inasmuch as malonate will almost completely inhibit pyruvate utilization in the absence of added oxalacetic or other dicarboxylic acids (9). Under these circumstances the synthesis of muscle  $\alpha$ -ketoglutarate in a radioactive bicarbonate medium would give rise to radioactive  $\alpha$ -ketoglutarate only if a process of exchange between intermediates (such as oxalacetate) in the reaction and the carbonate of the medium took place. If the quantity of radioactivity found in muscle  $\alpha$ -ketoglutarate were similar in magnitude to that found in the  $\alpha$ -ketoglutarate synthesized by liver, it would eliminate the necessity for proposing a stoichiometric utilization of  $\text{CO}_2$  in the latter process.

In an experiment with 7.6 gm. of minced pigeon muscle in 50



ml. of radioactive bicarbonate medium, the addition of 4.0 ml. of 0.5 M pyruvate and 6.0 ml. of 0.1 M fumarate led to the synthesis of 55.5 mg. of  $\alpha$ -ketoglutaric acid. The  $\alpha$ -ketoglutarate isolated as the hydrazone as described above contained no appreciable amount of radioactivity. This strongly suggests again that the process of CO<sub>2</sub> assimilation in pigeon liver is a metabolic reaction representing a stoichiometric utilization of inorganic carbonate.

#### DISCUSSION

Carbon dioxide participates in a variety of synthetic reactions in the tissues of higher animals: in the formation of urea (10-12), of carbaminohemoglobin (13), in the synthesis of  $\alpha$ -ketoglutarate from pyruvate in pigeon liver (1), and the formation of glycogen from lactic acid in the rat (14, 15). In the formation of urea, carbon dioxide is unquestionably used for synthetic purposes (*i.e.*, the reaction involves an increase in free energy) and apparently the physiological objective of the reaction, to eliminate metabolic end-products, has distracted attention from its similarity to the assimilation of CO<sub>2</sub> by the lower organisms (16). The more recent demonstrations of the metabolic utilization of carbon dioxide involve reactions suggesting that carbon dioxide, once formed, is not necessarily eliminated as such but can reenter the metabolism of the organism by several reaction paths to form a variety of tissue constituents. In such circumstances the exposure of the organism or cell to an environment containing radioactive carbon as CO<sub>2</sub> would lead to the presence of the tagged atoms in many of the compounds present in the tissue. If the appearance of radioactivity represented merely an exchange between the CO<sub>2</sub> of the medium and carboxyl groups of various organic acids, the use of radioactive CO<sub>2</sub> would not give any demonstration of a direct metabolic utilization of CO<sub>2</sub>. For the reasons outlined in the preceding sections of this paper such an explanation is regarded as unlikely for the appearance of radioactive carbon in  $\alpha$ -ketoglutaric acid synthesized in pigeon liver and it is believed that CO<sub>2</sub> participates directly as a reactant in this synthesis.

The mechanism by which carbon dioxide is utilized in  $\alpha$ -ketoglutarate synthesis in pigeon liver is unknown. The mechanisms already suggested (1, 5, 8) postulate the preliminary formation of oxalacetic acid from carbon dioxide and pyruvic acid, a reaction

for which there is as yet no direct experimental evidence. The condensation of oxalacetic acid with pyruvate to yield citrate as an intermediate can be excluded on the basis of the data presented in the present paper. The intermediate formation of isocitrate (5) rather than citrate by condensation of oxalacetate and pyruvate likewise seems improbable in view of the demonstrated equilibrium between citrate, isocitrate, and aconitic acid in most tissues (17).

#### SUMMARY

1. The synthesis of  $\alpha$ -ketoglutarate from pyruvate by minced pigeon liver involves the direct participation of  $\text{CO}_2$ . It is probable that 1 mole of  $\text{CO}_2$  is utilized for each mole of  $\alpha$ -ketoglutarate synthesized.

2. The radioactivity of the  $\alpha$ -ketoglutarate is confined entirely to the carboxyl group  $\alpha$  to the carbonyl group. This precludes the intermediate formation of citric acid, a conclusion further supported by experiments in which non-radioactive citrate was added to the tissue during the synthetic reaction.

3. A maximum of 1 in 5 carbon atoms of the  $\alpha$ -ketoglutarate is derived from the carbon dioxide of the medium.

4. The synthesized  $\alpha$ -ketoglutarate accounts for about one-fourth the assimilated  $\text{CO}_2$ . Release of part of this residual activity as  $\text{CO}_2$  by ninhydrin and chloramine-T suggests that it is present in part as amino acids or similar compounds.

5. The formation of  $\alpha$ -ketoglutarate in muscle does not involve the participation of carbon dioxide.

6. Evidence is presented that the process of  $\text{CO}_2$  assimilation in pigeon liver, as demonstrated by the use of radioactive  $\text{C}^{14}$ , is a metabolic reaction representing a stoichiometric utilization of inorganic carbonate.

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## A COLORIMETRIC OXIDATION-REDUCTION METHOD FOR THE DETERMINATION OF THE K VITAMINS\*

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A colorimetric method for the determination of the K vitamins has been devised on the basis of the oxidation-reduction titrations reported by Trenner and Bacher (2). The method involves a catalytic reduction of the quinone in butanol solution in the presence of phenosafranine as the indicator. The resulting vitamin hydroquinone is then treated with an excess of a butanol solution of 2,6-dichloroindophenol in the absence of air. The diminution in the color of the indophenol is a measure of the quinone originally present.

### *Reagents—*

**Butanol-acetate solution.** 500 mg. of reagent potassium acetate were dissolved in 50 cc. of water and the solution was diluted to 1 liter with acid-free *n*-butanol.

**Phenosafranine.** A stock solution containing 1 mg. of phenosafranine (Eastman, No. 1125) per cc. of water was prepared. 1 cc. of the aqueous solution was diluted to 100 cc. with *n*-butanol to give the reagent (10  $\gamma$  per cc.).

**Raney's nickel.** This was prepared according to Trenner and Bacher (2).

**2,6-Dichloroindophenol.** A stock solution was prepared by shaking 50 mg. of the Eastman material (No. P-3463) for 15 to 20 minutes in 100 cc. of butanol. The solution was filtered with suction through a dry filter paper. This stock solution, stored in a dark bottle in a refrigerator, was stable for at least 3 months.

\* This paper was presented before the meeting of the Federation of American Societies for Experimental Biology held at Chicago, April, 1941 (1).

1 cc. of this stock solution was diluted to approximately 50 cc. with the butanol-acetate solution. 10 cc. of this solution plus 5 cc. of the butanol-acetate solution gave an absorption of 85 per cent in the Evelyn colorimeter with Filter 660, when pure butanol was used to indicate 100 per cent transmission.

The variable amounts of salts, water, etc., in commercial samples of 2,6-dichloroindophenol make it impossible to state the

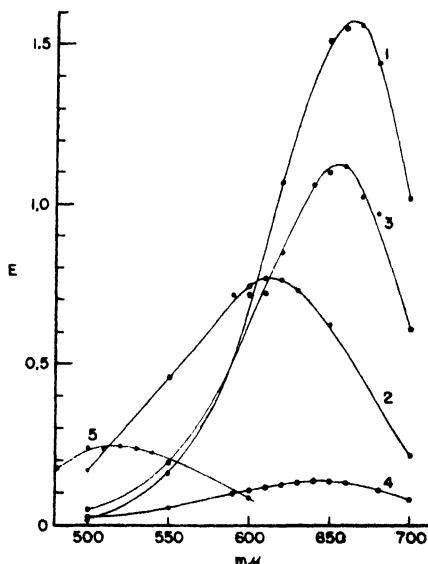


FIG. 1. Curve 1 shows the absorption data obtained with a 0.04 mm butanol solution of the 2,6-dichloroindophenol sodium salt; Curve 2, the same in aqueous solution; Curve 5, the same in aqueous solution at pH 5. After the aqueous solution of the indophenol sodium salt was extracted with an equal volume of butanol, the butanol phase gave the data shown in Curve 3, and the aqueous phase gave the data shown in Curve 4.  $E = \log I_0/I$ .

exact dilution of the stock solution. Different samples of the indophenol require slightly different dilution, which, however, may be followed colorimetrically. We have added the butanol-acetate solution until the reagent gave an absorption of exactly 85 per cent.

Filter 660 is used in the Evelyn colorimeter, since, as is shown in Fig. 1, maximum absorption of 2,6-dichloroindophenol is ob-

served at  $670\text{ m}\mu$  when the dye is dissolved in butanol. Curve 1 shows the absorption data obtained with a  $0.04\text{ mm}$  butanol solution of the indophenol sodium salt. This solution was titrated against 2-methyl-1,4-naphthohydroquinone according to Trenner and Bacher (2). Curve 2 shows the absorption data of a  $0.04\text{ mm}$  aqueous solution of the indophenol sodium salt. This solution was titrated according to Lorenz and Arnold (3). Curve 5 shows the absorption data of a  $0.04\text{ mm}$  aqueous solution of the indophenol at pH 5. The depression of the absorption and the shift of the peaks from right to left presumably correspond to the degree of hydrolysis of the indophenol sodium salt. Thus, butanol solutions of the indophenol, in contrast to aqueous solutions, are not only more stable, but exhibit greater spectral purity and show an optical density twice that of equivalent aqueous solutions.

The test is applicable to solutions containing 2 to  $10\text{ }\gamma$  of 2-methyl-1,4-naphthoquinone per cc. The colorimeter was calibrated against this material, since it is a crystalline solid and is more stable to light than vitamin  $K_1$ . The values are multiplied by 2.615 in order to convert them stoichiometrically to vitamin  $K_1$ . No attempt is made to differentiate vitamins  $K_1$  and  $K_2$  in this work. Unknown solutions of 2-methyl-1,4-naphthoquinone were analyzed, and the results checked the calibration curve within an error of 1.5 per cent with a maximum deviation of 5 per cent.

Trenner and Bacher (2) reported a factor of 1.08 in the stoichiometric comparison of 2-methyl-1,4-naphthoquinone and vitamin  $K_1$ . This factor is apparently a function of the dilution, since it was not observed by us in a series of twenty-two analyses of samples of pure vitamin  $K_1$  at concentrations ranging from 6 to  $24\text{ }\gamma$  per cc. In these experiments the average recovery was 100 per cent ( $\pm 3.5$ , maximum deviation 9 per cent).

*Apparatus*—The apparatus shown diagrammatically in Fig. 2 was constructed of glass with standard taper joints. It consists of two parts; a lower chamber A, in which the catalytic reduction is carried out, and an upper chamber B, in which the vitamin K hydroquinone partially reduces the standard indophenol reagent.

Within limits, the dimensions of the apparatus are arbitrary. Ours was constructed to permit the convenient reduction of  $7\text{ cc.}$  of solution in the lower chamber.  $5\text{ cc.}$  of the reduced solution

can then be pumped into the upper chamber. The upper chamber (12 mm., outside diameter, tubing) was calibrated at 15 cc. No doubt these volumes can be reduced considerably.

### Method

The entire apparatus is thoroughly cleansed with acetone and dried on a vacuum line. A small amount of the catalyst (about

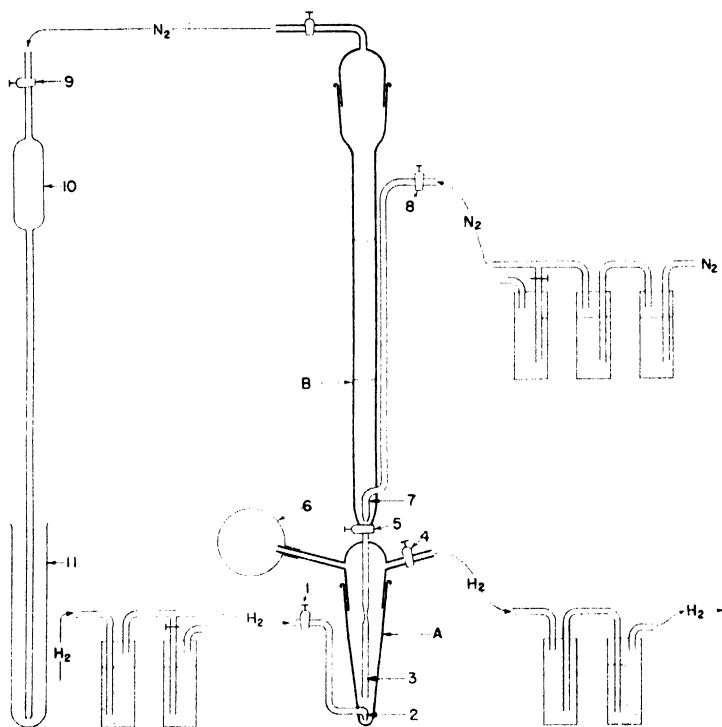


FIG. 2. Apparatus for the determination of the K vitamins

half the size of a pea) is placed in the bottom of the lower chamber. The sample is dissolved in the butanol-acetate solution and phenosafranine solution is added (0.5 cc. to 9.5 cc. of butanol-acetate). This solution is placed in the lower chamber together with the catalyst. A cotton plug is placed in the constricted delivery tube 3 and the apparatus is set up as shown. With stop-cocks 8, 5,

and 4 open and cock 1 closed, the air in the delivery tube 3 is replaced with nitrogen. Cocks 8 and 5 are closed, and 1 and 4 are opened.

Commercial hydrogen is passed through a spiral tower containing butanol to prevent evaporation losses. A water seal blow-off is placed in the line to prevent excessive pressures. The hydrogen is admitted through inlet 2 at a rate sufficiently brisk to keep the catalyst in motion. During this reduction period the rubber bulb 6 is squeezed from time to time to expel the air within it. After all the quinone is reduced, the phenosafranine is reduced and its pink color disappears. The reduction is continued for an additional 5 to 10 minutes to insure complete reduction.

While this reduction is in progress, 10 cc. of the standard indophenol reagent are pipetted into the upper chamber, and, as the reduction in the lower chamber approaches completion, the air in the indophenol solution is removed with a stream of nitrogen. This stream of commercial nitrogen is passed through a spiral tower containing alkaline hydrosulfite to remove traces of oxygen. The gas then passes through a butanol tower to saturate the gas. The gas train is by-passed with a butanol blow-off tower as a precautionary measure. The nitrogen is then admitted at inlet 7, bubbled through the indophenol solution for 10 minutes, and issues through the long stemmed, uncalibrated pipette 10 which is placed in the Evelyn colorimeter tube 11, as shown in Fig. 2.

After the reduction is complete, stop-cocks 1, 4, and 8 are closed. By means of the rubber bulb 6, the contents of the lower chamber are forced through the cotton filter (delivery tube 3) into the upper chamber as stop-cock 5 is opened. When the volume reaches the 15 cc. mark, cock 5 is closed and nitrogen is readmitted through cock 8 for 1 to 2 minutes to mix the contents of the upper chamber. Cocks 8 and 9 are then closed.

A deflated rubber bulb is placed on the end of the pipette, cock 9 remaining closed. The contents of the upper chamber are withdrawn by inserting the nitrogen-filled pipette into the chamber and slowly opening cock 9. When the solution is thus drawn up into the pipette, cock 9 is closed, and the rubber bulb is removed. The long stem of the pipette is then placed in the nitrogen-filled colorimeter tube, and the solution is run in without splashing. Readings are taken in the colorimeter exactly 3 minutes after



the hydroquinone and the indophenol are mixed. Atmospheric oxidation of the leucoindophenol does not occur under these conditions. Readings taken in the Evelyn colorimeter with Filter 660 remained constant for 40 to 60 minutes. Shaking or aerating reoxidizes the leuco base.

The pH of the solution to be tested is important. An accumulation of organic acids in an extract gives erroneous results. The reduction does not proceed smoothly in acid solutions, and, furthermore, these acids convert the indophenol sodium salt, either entirely or in part, to the red-colored free indophenol.

The pH of the butanol solutions can best be tested by mixing an aliquot of the sample with the indophenol reagent. If the unknown is acidic, a diminution in the blue color results, and this is evident in the colorimeter. The pH of acid samples can be corrected as follows: A 10 cc. portion of a petroleum ether solution of the unknown is shaken with an equal volume of cold half-saturated baryta solution. The ether layer is washed with 5 cc. of water, and again with 5 cc. of a 1:1 alcohol-water solution. The aqueous washes are extracted with 10 cc. of petroleum ether, and the combined ether extracts are dried over anhydrous sodium sulfate. The vitamin is transferred to butanol by adding butanol and distilling off the petroleum ether *in vacuo*. The recovery in a control series of these experiments was 98 per cent ( $\pm 2$ , maximum deviation 4 per cent).

The test was applied directly to several materials. Beef blood and urine were continuously extracted in the dark for 72 hours with petroleum ether. After adjustment of the pH the extracts were transferred to butanol and analyzed. Several oils were also examined without concentration. The data (items marked with a dagger) are listed in Table I. Although recoveries of added vitamin K<sub>1</sub> were satisfactory, the samples taken contained so little vitamin K that it could not be measured. The data shown are therefore limiting values; the actual vitamin content falls below these limits.

The presence of colored materials in the sample to be analyzed interferes with the performance of the test.<sup>1</sup> Furthermore, ex-

<sup>1</sup> Yellow concentrates convert the blue indophenol to a green-colored solution. This does not always interfere in the test, since the yellow is efficiently removed by color filters. If an aliquot of the unreduced un-

cessive amounts of extraneous, fat-soluble materials limit the size of the sample which can be tested. Consequently methods for the elimination of these materials were investigated.

Chromatographic separation of vitamin K<sub>1</sub> from extracts has been unsatisfactory in our hands, presumably, among other things, because of the photolability of the vitamin. It should be emphasized, that all steps performed in this work were carried out with a minimum exposure to light.

Fieser (8) introduced a remarkably simple method for the isolation of vitamin K<sub>1</sub> from alfalfa concentrates. He reduced the

TABLE I  
*Vitamin K<sub>1</sub> Content of Various Materials*

Sample	Vitamin K <sub>1</sub> in dry tissue	Recovery of added vitamin K <sub>1</sub>	Data approximated from published bioassays*
	<i>γ per gm.</i>	<i>per cent</i>	<i>γ per gm.</i>
Alfalfa .....	15.6	92	16 (4)
Beef liver .....	6.9	102	8 (5)
Fresh sardine meal .....	2.9	94	Variable with history of sample (6)
	<i>γ per cc.</i>		
Beef blood† .....	<0.26	98	Very low (7)
Human urine† .....	<0.075	90	
Cod liver oil† .....	<2.0	96	
Peanut oil† .....	<10.0	96	

\* The figures in parentheses indicate bibliographic references.

† Examined without concentration.

concentrate with sodium hydrosulfite and transferred the concentrate to petroleum ether. The vitamin hydroquinone was then removed from the ethereal solution with Claisen's alkali. The potassium salt of the hydroquinone, which was formed in the alkaline phase, was hydrolyzed by the mere addition of water, and the free vitamin hydroquinone was then extracted with petroleum ether. This procedure cannot be used as such in conjunction with the present test, but we have devised a modified procedure

known, upon mixing with the indophenol, does not alter the transmission by more than a dilution factor, the color in the unknown will probably not interfere.

based on the same principle which overcomes the difficulty. This method is carried out in the apparatus shown diagrammatically in Fig. 3. Although the dimensions of the apparatus are more or less arbitrary, ours was constructed to contain a total volume of 100 cc. of solution.

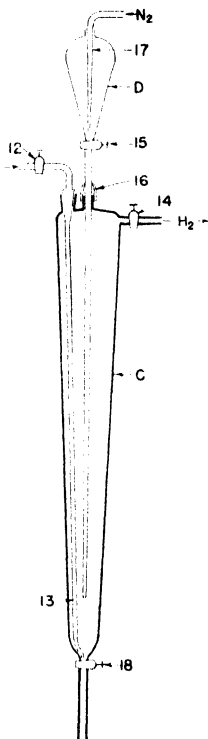


FIG. 3. Apparatus for the separation of the vitamin K hydroquinones from colored extracts by means of the reductive Claisen's alkali treatment.

The neutral sample, containing 1 to 2 gm. of solids or oils, is dissolved in a mixture of 5 cc. of methanol and 10 cc. of petroleum ether, and 10 to 15 mg. of powdered phenosafranine are added. The solution is placed in the lower chamber *C*, and a charge of Raney's nickel (about the size of a pea, or larger if the reduction is too slow) is added. The hydrogen is led through a methanol tower to prevent consequent evaporation losses before it is ad-

mitted through stop-cock 12. The hydrogen is admitted through inlet 13 at a rate sufficiently brisk to keep the catalyst in motion. The large amount of phenosafranine makes it relatively simple to follow the reduction in spite of the high color of some concentrates. The reduced phenosafranine also protects the vitamin hydroquinone from accidental atmospheric oxidation. A leakage of air into the system, which vitiates the results, is readily detected by the return of the color of the phenosafranine.

After the reduction is complete, 15 cc. of Claisen's alkali (50 gm. of potassium hydroxide in 25 cc. of water diluted to 100 cc. with methanol) are placed in the upper chamber *D*. Air is removed by bubbling nitrogen from inlet 17 through this solution—a process usually requiring about 10 minutes. The Claisen's alkali is then admitted through stop-cock 15 and the contents of the lower chamber are mixed by readmitting the hydrogen stream through stop-cock 12. The potassium salt of the vitamin hydroquinone is thus formed and remains in the alkaline phase.

The hydrogen supply is then cut off by closing cock 12. Cock 14 is left open during this operation. The system is protected from the entrance of air by connecting cock 14 with a trap such as shown connected to cock 4 in Fig. 2. The two phases are allowed to separate. The upper chamber *D* is then raised through the greased rubber collar 16 so that the petroleum ether can be removed by connecting an aspirator to the mouth of the upper chamber. This washing procedure, which serves to remove neutral fats, vitamin E, and ether-soluble chromogens, may be repeated as necessary.

After the petroleum ether is removed, 30 cc. of thoroughly gas-free water are admitted through stop-cock 15 to hydrolyze the potassium salt of the vitamin K hydroquinone. After the material is mixed for 5 minutes, 30 cc. of gas-free petroleum ether are run in through cock 15 to extract the hydroquinone. After the material is mixed for 10 minutes with hydrogen, the two phases are allowed to separate, and the highly colored, aqueous, alkaline, lower layer, now free of vitamin K, is drawn off through stop-cock 18. The petroleum ether layer, which contains the hydroquinone, is then washed in the apparatus with 30 cc. of gas-free water which are subsequently removed by way of cock 18. The petroleum ether is run into a graduated cylinder and its volume

is measured, and the sample is dried over anhydrous sodium sulfate. At this point, the hydroquinone is exposed for the first time to air oxidation. The small amount of phenosafranine in the petroleum ether becomes pink, but is adsorbed by the sodium sulfate, leaving practically colorless solutions. The vitamin is then transferred to butanol, as described above. This solution is then analyzed as usual. No pH adjustment is necessary following this procedure.

In a control series of fifteen experiments, the average recovery of added vitamin K<sub>1</sub> was 83 per cent ( $\pm 3$ , maximum deviation 10 per cent). Consequently values obtained following this procedure are divided by the factor 0.83. We believe this correction factor is essentially a distribution coefficient. It expresses, among other things, the ratio of the amounts of the hydrolyzed hydroquinone in the petroleum ether and alkaline phases. We have restricted the amount of solids (or oils) in the test sample to 1 or at the most 2 gm., since excessive amounts of these ether-soluble materials cause emulsification and variation in the phase volumes. By restricting the solids to 1 to 2 gm. the distribution coefficient has remained constant, as judged by recovery experiments involving added vitamin K<sub>1</sub>.

This treatment has been used in the analysis of the samples recorded in Table I. Extracts were prepared as follows: Samples were dehydrated by refluxing for 3 hours with alcohol. The solids, removed by filtration, were extracted for 48 to 96 hours in a Soxhlet apparatus with petroleum ether (b.p. 60–80°). The combined alcohol-ether extracts were concentrated to dryness *in vacuo*, under nitrogen, and the residue was taken up in a minimal volume of petroleum ether. The pH was adjusted and the solution was diluted to contain 2.5 to 15  $\gamma$  of vitamin K<sub>1</sub> per cc. 10 cc. of this solution, containing less than 2 gm. of solids or oils, and 5 cc. of methanol were then submitted to reduction and treatment with Claisen's alkali as described above. After the vitamin K<sub>1</sub> hydroquinone was separated from interfering materials and colors, the hydroquinone was reoxidized to the quinone in the presence of air. The sample was then transferred to butanol and analyzed as usual. Readings were taken *exactly* 3 minutes after the sample and the 2,6-dichloroindophenol in the upper chamber of the test apparatus were mixed. The data obtained are consistent with

previously published values obtained by biological assay procedures, and the recoveries of added vitamin K<sub>1</sub> are within the limits of error of the procedure. The data are expressed in terms of vitamin K<sub>1</sub>, since no attempt is made to differentiate vitamins K<sub>1</sub> and K<sub>2</sub> in the present procedure.

#### DISCUSSION

Concerning the specificity of the method described here, Trenner and Bacher (2) showed that the potential of phenosafranine lies well below that of any of the known quinones, and that the hydroquinones all reduce 2,6-dichloroindophenol. Thus, any substance having an oxidation-reduction potential above that of phenosafranine and about 150 millivolts below that of the indophenol will be quantitatively measured in the present procedure. The group of substances falling within this range, however, is cut down considerably by the means used to extract vitamin K from natural materials; for example, the sugars, thiols, ascorbic acid, etc., are not soluble in petroleum ether.

The use of the preliminary treatment of the hydroquinone with Claisen's alkali adds markedly to the specificity of the test. Vitamin K<sub>1</sub> hydroquinone is soluble in Claisen's alkali, but not in aqueous alkali. These cryptophenolic properties are generally associated with polysubstituted hydroquinones which possess fat-soluble groupings. As a consequence of these properties, it is possible to wash a Claisen's alkali solution of the vitamin hydroquinone with petroleum ether without removing the vitamin K<sub>1</sub> hydroquinone.<sup>2</sup> The simple addition of water to the ether-washed Claisen's alkali, however, hydrolyzes the potassium salt of the vitamin K<sub>1</sub> hydroquinone, thus making it possible to extract the hydroquinone with petroleum ether. And this petroleum ether extract can then be analyzed for vitamin K<sub>1</sub> as usual.

It should be noted that after the hydrolysis of the potassium salt of vitamin K<sub>1</sub> hydroquinone, by the addition of water, the aqueous phase remains approximately 10 per cent in potassium hydroxide and 25 per cent in alcohol. Hence the alkaline phase will retain many hydroquinones. For example, the benzoquinones

<sup>2</sup> This treatment will remove the cryptophenolic vitamin E, which does not form a potassium salt even in Claisen's alkali.

(with the possible exception of duroquinone) and the naturally occurring 3-hydroxynaphthoquinones, such as phthiocol, lapachol, hydrolapachol, and lumatiol, give hydroquinones which are not extracted along with the vitamin K<sub>1</sub> hydroquinone, but, on the contrary, remain in the diluted Claisen's alkali. Similarly, 2-methyl-1,4-naphthoquinone is quantitatively separated from the vitamin by this procedure. The tocopherylquinones, however, are not separated from the K vitamins.

When readings are taken within 3 minutes after the hydroquinone and the indophenol are mixed in the test procedure, the influence of extraneous, slowly reducing substances is eliminated. For example, the tocopherylquinones, or the tocopherylhydroquinones to which they are converted during the Claisen's alkali procedure, are extracted along with the vitamin K hydroquinone. However, the reduction of the 2,6-dichloroindophenol by  $\alpha$ - and  $\beta$ -tocopherylhydroquinones proceeds very slowly, reaching a steady maximum only after 40 to 60 minutes. Consequently, readings taken 3 minutes after the indophenol and hydroquinone solutions are mixed are not significantly altered by the presence of the amounts of tocopherylquinones usually found in extracts of natural materials.

It may be noted here that the tocopherylquinones are quantitatively measured by the test procedure if the final steady readings are taken 40 to 60 minutes after the tocopherylhydroquinones are mixed with the indophenol reagent. The same apparatus and the same calibration curve established with 2-methyl-1,4-naphthoquinone are applicable. The final values are to be multiplied by the stoichiometric factor of 2.5 for conversion to  $\alpha$ -tocopherol.

The continued drop in some of our readings indicated that there was a substance or substances like the tocopherylquinones present in some of our extracts. Whereas these substances are eliminated satisfactorily by restricting the reading time, it was deemed desirable to investigate other means of elimination. The lability of vitamin K to alkali and acids suggested that duplicate determinations before and after destruction of the vitamin K might give an accurate estimate of the vitamin. Destruction of the vitamin with alkali has not been readily amenable to standardization in our hands. Treatment of petroleum ether extracts with 85 per cent sulfuric acid as in the method of Parker and McFarlane

(9) removed all of the vitamin K, but this treatment, which also removes the tocopherylquinones, removed all of the slowly reducing materials as well as the K vitamins.

These slowly reducing substances exhibit the properties of the tocopherylquinones; *viz.*, they are fat-soluble; they pass through the Claisen's alkali treatment; they are removed by the sulfuric acid treatment; their oxidation-reduction potential falls within the required range; and the rate at which their hydroquinones reduce the indophenol reagent is characteristic. If the presence of the tocopherols in these extracts could be demonstrated, this would augment the evidence that these slowly reducing substances are tocopherylquinones. This has been demonstrated with our alfalfa extract, as follows:

An aliquot of our alfalfa extract was run through the reductive treatment with Claisen's alkali and analyzed for vitamin K, the appropriate reading being taken at 3 minutes. (This gave results in essential agreement with bioassays published elsewhere and good recoveries of added vitamin K<sub>1</sub> were obtained.) Readings were taken again after 60 minutes. Assuming that the distribution coefficients of the vitamin K hydroquinones and the tocopherylhydroquinones are essentially equal, the tocopherylquinone content of the aliquot was measured in terms of 2-methyl-1,4-naphthoquinone by subtracting the initial from the final reading. Multiplying this by the stoichiometric factor 2.5 showed that the sample contained about 15  $\gamma$  of tocopherylquinone per gm. of dry alfalfa.

A second aliquot of this same alfalfa extract was treated with sulfuric acid according to the method of Parker and McFarlane. This removes the K vitamins and the tocopherylquinones. The residue was then oxidized with gold chloride by the method of Karrer *et al.* (10). After removal of the gold, the tocopherylquinone was analyzed as usual. The sample contained about 12  $\gamma$  of tocopherol per gm. of dry alfalfa.

From the foregoing it would appear that with only minor manipulations of the samples to be analyzed, the same apparatus and the same procedure used for the determination of the K vitamins can be used for the determination of the tocopherols and the tocopherylquinones.



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## SUMMARY

A colorimetric, oxidation-reduction method for the determination of the K vitamins has been described. The test is performed in butanol solution. This increases the stability and spectral purity and doubles the optical density of the 2,6-dichloroindophenol reagent. The method is applicable to solutions containing as little as 5  $\gamma$  of vitamin K<sub>1</sub> per cc. A means for applying the method to colored extracts has been presented. The vitamin K content of a number of materials has been reported. The specificity of the method has been discussed, and it has been shown that vitamin K<sub>1</sub> can be differentiated from 2-methyl-1,4-naphthoquinone and the tocopherylquinones. Evidence has been presented to show that extraneous, slowly reducing substances present in certain extracts are tocopherylquinones. The method is quantitatively applicable to the determination of the tocopherylquinones and the tocopherols, after oxidation to the corresponding quinones.

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# PREPARATION OF *l*-ALANINE FROM *dl*-ALANINE BY THE ACTION OF *d*-AMINO ACID OXIDASE

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In 1935 Krebs (1) demonstrated the presence in kidney tissue of an enzyme which specifically oxidizes amino acids of "unnatural" (*d*) configuration, leading to the formation of ammonia and  $\alpha$ -keto acids. This enzyme has been utilized by Lipmann and coworkers (2) to examine the contention of Kögl and Erxleben (3) that tumor tissue is specifically characterized by the presence of amino acids of "unnatural" (*d*) configuration. Since this enzyme does not attack the natural (*l*) amino acids, it has been found useful for the preparation of *l*-amino acids from the corresponding *dl* substances (Duschinsky and Jeannerat (4)). In making use of *d*-amino acid oxidase for this purpose, we found it possible to simplify the experimental technique and are therefore presenting a detailed description of our procedure. The preparation of considerable quantities of *l*-amino acids may readily be accomplished by this method. Its application to the preparation of *l*-alanine is here described.

## EXPERIMENTAL

*d*-Amino Acid Oxidase—A dry, stable enzyme source was prepared in the following manner. Fresh frozen sheep or hog kidneys were ground into 8 volumes of acetone at room temperature (up to 24°). The material was stirred well and allowed to stand 2 hours. The supernatant fluid was removed by decantation, and the material was filtered on coarse paper. The precipitate was resuspended in 4 volumes of acetone and again filtered. The acetone treatment was repeated once more. The period from the time that the material is ground to the completion of the acetone treatment should not exceed 6 hours. The precipitate thus obtained was

freed of excess acetone in a vacuum desiccator over  $\text{CaCl}_2$  or  $\text{H}_2\text{SO}_4$  or in a vacuum oven at room temperature for 6 to 18 hours. The dry kidney powder thus prepared may be stored in the cold for some months without appreciable deterioration.

*Preparation of l-Alanine*—120 gm. of dry, finely powdered kidney were thoroughly mixed with 600 cc. of water. Water was added to about 3200 cc. and the material was stirred at 35–40° for 30 minutes. The coarse precipitate was removed by filtering through fine cheese-cloth. The cloudy solution remaining was quickly passed through a Sharples supercentrifuge, whereby an almost clear solution was obtained. If facilities permit, centrifuging the entire suspension at 2000 to 2500 R.P.M. may be substituted for the filtration and supercentrifugation. The extraction of the kidney powder must be completed to this point within 1½ hours.

The enzyme solution thus obtained was immediately added to a solution of 72 gm. of dl-alanine in 40 cc. of N NaOH and 760 cc. of water, and oxygen was bubbled through the solution from a sintered glass plate with very vigorous stirring. The stirring and oxygenation were continued for a period of 5 hours, during which the temperature was maintained at 35–40°. The total oxygen uptake, which may be measured with a closed system, should be 4600 cc. The reaction should be complete in 3 to 5 hours.

The solution was treated with 40 cc. of N HCl, heated to boiling to coagulate the dissolved protein material, and filtered. The clear filtrate was concentrated *in vacuo* to 600 to 900 cc. Sufficient 20 to 25 per cent aqueous trichloroacetic acid was added to bring the concentration of the entire solution to 2.5 per cent. The slight precipitate was removed by vacuum filtration through coarse paper and a thin layer of Filter Cel, 100 cc. of concentrated HCl were added, and the solution was evaporated to dryness *in vacuo*. The evaporation was repeated two times with the addition of small portions of water to remove excess HCl and then three times with the addition of small portions of alcohol. The precipitate remaining was extracted with 200 cc. of hot absolute alcohol. The residue (predominantly  $\text{NH}_4\text{Cl}$  and  $\text{NaCl}$ ) was reextracted with 100 cc. of 95 per cent alcohol. To the combined filtrates were added 67 cc. of aniline with stirring. The material was cooled to about 20° during ½ to 1 hour and filtered with suction. The brown precipitate thus obtained was suspended in 100 cc. of warm alcohol,

filtered, and washed on the filter with 50 cc. of ether. 33 to 40 gm. of crude *l*-alanine were thus obtained.  $[\alpha]_D^{25} = +9-12^\circ$  (3 per cent in HCl). This material contains a considerable amount of an inactive substance. The alanine was recrystallized from 3 parts of boiling water by addition of 9 parts of alcohol. In the recrystallization any material that is insoluble in 3 parts of boiling water is not alanine and may safely be discarded. Yield, 22 gm.,  $[\alpha]_D^{25} = +14.5^\circ$ ; amino N, 15.5 per cent; calculated for  $C_3H_7O_2N$ , 15.7 per cent. After one more recrystallization the rotation was  $[\alpha]_D^{25} = +14.5^\circ$ .

#### SUMMARY

Detailed directions are given for the preparation of *d*-amino acid oxidase and its use in the preparation of *l*(+)-alanine from *dl*-alanine.

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# FACTORS INFLUENCING RECOVERY OF INJECTED LABELED PHOSPHORUS IN VARIOUS ORGANS OF THE RAT

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Despite the volume of work that has appeared on radioactive phosphorus as an indicator for metabolic processes, no satisfactory evidence has so far been provided for the establishment of what may be termed the "safe tracer dose" with respect to the volume of solution and the amount of labeled phosphorus to be injected. Such information is necessary for the interpretation of results obtained from different sized animals treated with varying doses of the labeling agent. From the following series of experiments it will be shown that, within the limits studied, neither the number of mg. of  $\text{Na}_2\text{HPO}_4$  nor the volume of the injected  $\text{Na}_2\text{HPO}_4$  solutions influences the recovery of total phosphorus in various tissues.

## EXPERIMENTAL

*Determination of Total Radioactive Phosphorus*—In the following series of experiments the total labeled phosphorus recovery from samples of brain and blood was determined by a combined wet and dry ashing procedure. The tissue was placed in a tared porcelain crucible fitted with a cover. After weighing, an excess of  $\text{Mg}(\text{NO}_3)_2$  solution was added, together with 1 cc. of concentrated nitric acid. This was heated on the steam bath, with frequent additions of nitric acid, until the tissue was digested. Owing to the high fat content of the brain, some fat usually remained undigested. At least 1 cc. of concentrated hydrochloric acid was then added and the contents dried. The hydrochloric acid serves

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to decompose the excess nitric acid and thus prevents puffing of the material during the ensuing dry ashing. The crucibles were placed in a cold muffle and gradually heated to 150–200°. If any puffing was observed, a few drops of concentrated hydrochloric acid were cautiously added. The temperature was then raised to 550°, and the ashing was usually complete within 2 hours. After cooling, the ash was dissolved in concentrated HCl. If further ashing was necessary, the contents of the crucibles were dried and the dry ashing repeated. Otherwise the dissolved ash was made up to volume and a suitable aliquot pipetted onto a blotter. If the activity in the sample was low, the whole sample was mounted on the blotter directly from the crucible. It was found advisable to add 0.5 cc. of concentrated  $\text{NH}_4\text{OH}$  to these blotters to neutralize the HCl; if this was not done, the blotters decomposed while being dried. When dry, the blotters were wrapped in cellophane and the radioactivity determined as previously described (1). The samples of liver, kidney, and muscle were not ashed. These tissues were reduced to a homogeneous mixture in an agate mortar. Duplicate samples of approximately 100 mg. were weighed on small squares (1 cm.  $\times$  1 cm.) of waterproof cellophane. The cellophane squares were placed directly on a blotter. The blotter was then wrapped with waterproof cellophane. With a glass vial as a roller, the tissue was spread uniformly over the surface of the blotter. The blotter was then dried overnight under an electric light. The radioactivity was determined in the usual manner. Trial determinations showed that values obtained by this method of measuring total phosphorus recovery agree very closely with values by the method of ashing. This method can be conveniently applied to an isotope possessing powerful radiation when the amount of self-absorption is negligible.

*Series 1*—Twenty rats of uniform weight, each weighing 50 gm., were used here. They were divided into four groups of five animals. Each rat of Group I received subcutaneously 0.25 cc. of a  $\text{P}^{32}$  solution that consisted of 0.10 cc. of isotonic radioactive  $\text{Na}_2\text{HPO}_4$  solution (3 mg. of P per cc.) and 0.15 cc. of isotonic NaCl solution. Those of Group II each received 0.50 cc. of a  $\text{P}^{32}$  solution made up by the addition of 0.10 cc. of an isotonic radioactive  $\text{Na}_2\text{HPO}_4$  solution to 0.40 cc. of an isotonic NaCl solu-

tion. The rats of Group III were injected with 1.00 cc. of a  $P^{32}$  solution that was prepared by adding 0.10 cc. of an isotonic radioactive  $Na_2HPO_4$  solution to 0.90 cc. of isotonic NaCl. In Group IV each rat received 2.00 cc. of a  $P^{32}$  solution made up by the addition of 0.10 cc. of an isotonic radioactive  $Na_2HPO_4$  solution and 1.90 cc. of isotonic sodium chloride. The rats were killed 24 hours after the  $P^{32}$  injection and the recovery of total labeled phosphorus in whole brain and whole blood determined.

*Series 2*—Six 15 gm. rats, six 50 gm. rats, and six 200 gm. rats were used in this experiment. In this series the volume injected was graded according to the weight of the animal, but the amount of labeled phosphorus injected was not allowed to vary. Each rat received subcutaneously 1 cc. of a  $P^{32}$  solution per 100 gm. of body weight. Each animal, irrespective of size, received exactly 0.10 cc. of labeled isotonic  $Na_2HPO_4$  solution; the remainder of the solution injected was isotonic NaCl. These animals were killed 24 hours later and the recovery of total labeled phosphorus in brain and whole blood determined.

*Series 3*—Five 15 gm. rats, five 50 gm. rats, and five 200 gm. rats were used in this experiment. These animals received subcutaneously graded doses of an isotonic labeled  $Na_2HPO_4$  solution in the proportion of 1 cc. of solution per 100 gm. of body weight. In this series, therefore, the amount of phosphorus injected was allowed to vary according to body weight. This experiment should be contrasted with that of Series 2, in which each rat received exactly the same amount of phosphorus regardless of its size. 24 hours later the rats were killed and the recovery of total labeled phosphorus in liver, kidney, skeletal muscle (gastrocnemius), brain, and blood determined.

*Effect of Variation in Volume of  $P^{32}$  Solution Administered*—The results obtained by the administration of *different volumes* of  $P^{32}$  solution, all containing, however, the *same amount of labeled phosphorus*, namely 0.3 mg. per rat, are shown in Table I. The results were obtained from the experiment on Series 1. No differences were found in the recovery of radioactive phosphorus in brain and blood at 24 hours despite the variations in the volumes injected. While the total phosphorus recovery in the brain, a tissue which is slowly penetrated by phosphorus (2-5), is found to be the same regardless of the volume injected, it seems reason-



able to assume that the same result would be found for a tissue which is more rapidly penetrated.

*Effect of Variation in Amounts of Labeled  $\text{Na}_2\text{HPO}_4$  Administered*—The results recorded in Table II were obtained from the

TABLE I

*Effects of Variation in Volume Injected upon Recovery of Labeled Phosphorus in Whole Brain and Whole Blood of 50 Gm. Rats*

All values are expressed as per cent of the administered labeled phosphorus. Each value represents the average of five closely agreeing results.

Volume injected cc.	Brain		Blood per gm.
	Per whole organ	Per gm.	
0.25	0.213	0.163	0.226
0.50	0.222	0.175	0.225
1.00	0.221	0.172	0.226
2.00	0.209	0.163	0.215

TABLE II

*Effects of Variation in Amount of Phosphorus Administered upon Recovery of Labeled Phosphorus in Brain and Blood*

All recovery values are expressed as per cent of the administered labeled phosphorus. Each value represents the average of five or six closely agreeing results.

	15 gm. rats		50 gm. rats		200 gm. rats	
	Series 2	Series 3	Series 2	Series 3	Series 2	Series 3
Volume injected, cc. . . . .	0.15	0.15	0.50	0.50	2.00	2.00
P injected, mg. . . . .	0.30	0.45	0.30	1.50	0.30	6.00
Recovery in brain						
Per whole organ . . . . .	0.888	0.857	0.222	0.227	0.105	0.109
" gm. . . . .	1.07	1.07	0.175	0.165	0.0648	0.0634
Recovery in blood per gm. . . . .	0.707	0.705	0.222	0.222	0.111	0.117

experiments on Series 2 and 3. The 15 gm. rats of Series 3 received 1.5 times as many mg. of phosphorus as did the 15 gm. rats of Series 2; yet the recovery of radioactive phosphorus in brain and blood was practically identical in both these groups. The 50 and 200 gm. rats yielded essentially the same results. Despite the fact that the 200 gm. rats of Series 3 received 20 times

as much phosphorus as did the 200 gm. rats of Series 2, the recoveries of radiophosphorus in brain and blood of both series were practically the same.

*Comparison of Phosphorus Uptake per Gm. and per Whole Organ in Animals of Various Sizes*—Table III shows the recovery of total labeled phosphorus for several tissues, calculated per gm. and per whole organ. These results were obtained from the experiment on Series 3, in which the amount of phosphorus administered per unit of body weight was kept constant. Total skeletal muscle was estimated from Jackson and Lowry's data (6); ac-

TABLE III

*Comparative Uptake of Labeled Phosphorus by Whole Organs and per Gm. of Tissue in Animals of Various Weights 24 Hours after Injection*

All values are expressed as per cent of the administered labeled phosphorus. Each value represents the average of five closely agreeing results.

Rat weight	Per whole organ					Per gm.				
	Liver	Kidney	Brain	Whole blood	Muscle	Liver	Kidney	Brain	Whole blood	Muscle
gm.										
15	3.21	0.825	0.857	0.85*	11†	6.34	4.51	1.07	0.705	3.31
50	4.11	0.747	0.227	0.80*	15†	1.93	1.46	0.165	0.222	0.885
200	5.97	0.927	0.109	1.5*	16†	0.84	0.595	0.0634	0.117	0.190

\* Estimated from Donaldson (7).

† Estimated from Jackson and Lowry (6).

cording to these workers, skeletal muscle represents 23 per cent of the body weight of the 15 gm. rat, 33 per cent in the 50 gm. rat, and 43 per cent in the 200 gm. rat. Donaldson's data (7) were used to estimate the recovery of phosphorus in whole blood of the entire rat; according to him, blood represents 8 per cent of the body weight of the 15 gm. rat, 7 per cent of the 50 gm. rat, and 6 per cent of the 200 gm. animal.

Table III shows that for most of the tissues examined the percentage of the administered radiophosphorus recovered per whole organ remains constant or increases slightly as the animal increases in size. Increases in rat sizes from 15 to 50 gm. and from 15 to 200 gm. represent a 3- and 13-fold increase; yet the percentage of the administered radioactive phosphorus recovered in the whole

*kidney* did not differ significantly in the 15, 50, and 200 gm. rats. Accepting the above estimates of total muscle in rats of different sizes, we conclude from Table III that as the rat grows larger only a moderate increase occurs in the uptake of radiophosphorus by the *entire* skeletal muscle. The retention of radiophosphorus by the entire volume of blood of each animal was calculated in a similar manner. A slight rise was again observed in the amount of radiophosphorus retained as the animal grew larger. The recovery in whole liver rose from 3 to 6 per cent as the rat's weight changed from 15 to 200 gm.

The brain differed strikingly from liver, kidney, blood, and muscle in that the percentage of the administered radioactive phosphorus recovered in the *whole brain* decreased as the animal grew larger. When the uptake of  $P^{32}$  in the whole brain of the 15 gm. rat was compared with that in the 200 gm. rat, an 8-fold decrease was observed.

The deposition of the administered labeled phosphorus *per gm. of tissue* has also been examined in Table III. The recovery per gm. was smaller in all tissues of the 200 gm. rat than in the 15 gm. animal. When the recoveries of  $P^{32}$  per gm. of tissue are compared in the 15 and 200 gm. rats (Table III), 6- to 8-fold decreases for liver, kidney, and whole blood and a 17- to 18-fold decrease for muscle and brain are found.

#### DISCUSSION

The finding that large variations in the amount of  $Na_2HPO_4$  injected produced no change in the percentage of the administered labeled phosphorus recovered in brain and blood (Table II) is perhaps not surprising. Although appreciable amounts of labeled phosphorus were injected, they represent small fractions of the total phosphorus already present in the animal. The total amount of phosphorus within the rat is not appreciably altered by the amount of labeled phosphorus introduced in the present study.

When comparisons of recoveries of injected radiophosphorus are being made among different organs (for example, liver and kidney) either in the same animal or in animals of the same weight, such comparisons are most conveniently made per gm. of tissue. But when recoveries by the *same organ* are being measured in animals of different sizes, the question arises whether comparisons

should be made per whole organ or per gm. of tissue. The observation that in animals of different sizes the recoveries of the injected radiophosphorus in any given organ are more nearly alike when compared on the basis of the whole organ than when compared per gm. of tissue is of importance in the interpretation of results involving labeled isotopes. The data of Table III as well as those of Table IV support the view that recovery per whole organ should be considered when phosphorus and phospholipid recoveries by tissues obtained from animals of different sizes are compared. In Table IV the labeled phospholipid and the total labeled phosphorus recoveries by several tissues of the adult rat (200 gm.) and the adult mouse (23 gm.) are compared. Table IV shows that even in these two different animals<sup>1</sup> the recoveries of the injected phosphorus as phospholipid and total phosphorus agree quite closely when compared on the basis of the whole organ. On the other hand, if the comparisons of the same organ are based on the recovery per gm. of tissue, the activity in the smaller animal is many times greater than in the larger (Tables III and IV). It is difficult to believe that the activity of muscle, liver, or kidney can be so much greater in the mouse than in the rat, or even that the activities of these tissues should be greater in the young rat than in the old.

The above conclusions are shown to hold fairly well for liver, kidney, muscle, and blood. There was a 13-fold change in weight from the youngest to the oldest rat investigated; yet for each tissue the maximum change in recovery of labeled phosphorus per whole organ was less than 2-fold.

In the case of the brain it was found that the recovery of total labeled phosphorus continues to decrease as the rat grows larger, even though comparisons are made on the basis of the whole organ. From the 15 to the 200 gm. rat this decrease was 8-fold. Per gm. of tissue the decrease in recovery was even greater; namely,

<sup>1</sup> The time of maximum recovery of  $P^{32}$  for a given organ may vary somewhat in rat, mouse, etc. This variability may be significant in tissues like liver and kidney, in which maximum recoveries occur early. The intervals chosen in the present study, namely 24 and 48 hours, are close to the time when maximum recoveries are found (2, 5). Despite this consideration, however, the data of Table IV suggest that when  $P^{32}$  recovery by the same organ in mouse and rat is compared the recovery per whole organ is to be preferred to recovery per gm. of tissue.

TABLE IV  
*Phospholipid and Total Phosphorus Recovery of Liver, Muscle, Brain, Whole Blood, and Kidney at 24 and 48 Hour  
 Interval after Administration of Labeled Phosphorus*  
 All values are expressed as per cent of the administered labeled phosphorus.<sup>†</sup>

Activity	Time interval	Animal	Weight	Liver		Muscle		Brain		Blood		Kidney	
				Whole organ	Per gm.	Whole organ	Per gm.	Whole organ	Per gm.	Whole organ	Per gm.	Whole organ	Per gm.
Phospholipid	24 hrs.	Mouse	23	2.3 (8)	1.9 (8)			0.033*	0.15*				
		Rat	200	1.8 (1)	0.30 (1)			0.021 (9)	0.013 (9)				
Phospholipid	48	Mouse	23	1.2 (8)	0.97 (8)	1.3 (8)	0.20 (8)	0.035*	0.16*				
		Rat	200	1.3 (1)	0.20 (1)	0.8 (10)	0.007 (10)	0.034 (9)	0.021 (9)				
Total P	24	Rabbit	2000					0.030	0.0037				
		Mouse	23	5.1 (5)	4.2 (5)	22 (5)	3.2 (5)	0.11 (5)	0.51 (5)	1.1 (5)	0.73 (5)	0.72 (5)	4.0 (5)
		Rat	200	6.0	0.84	16	0.19	0.11	0.063	1.5	0.12	0.93	0.60

The numbers in parentheses refer to the source of data.

\* Jones, H. B., and Chaikoff, I. L., unpublished data.

18-fold. Despite this change with age it should nevertheless be noted that the recoveries per whole brain in *adult* mouse and rat agree very closely (Table IV).

The growth of the brain differs strikingly from that of other tissues in that it is characterized by a very rapid early phase and a slow one thereafter. Although the other tissues here investigated also have an early phase of rapid growth, they continue to grow uniformly thereafter (7). This may explain why the recovery of radiophosphorus by the brain differs from that by the other tissues. It seems unlikely, however, that this is the sole explanation of the decreased recovery found in the older rat. Despite the fact that the various brain divisions, namely forebrain, cerebellum, medulla, and spinal cord, show great differences in growth rates, it is shown in the next paper (11) that all these divisions exhibit the same 7- or 8-fold drop in phosphorus recovery per whole organ as the animal grows in size. For example, the forebrain in the 15 gm. rat amounts to 4 per cent of the body weight, while in the 200 gm. rat it represents 0.6 per cent, a relative decrease of 7 times. The spinal cord in the 15 gm. rat is 0.4 per cent of the body weight and in the 200 gm. rat 0.2 per cent, a relative decrease of only 2 times (9).

The chemical changes associated with the growth of the brain may also account, in part, for the  $P^{32}$  recovery by the brain. While the growth of the liver, kidneys, and muscles, etc., involves the proliferation of more cells of like chemical make-up, the growth of the brain after birth involves almost no cell division, but instead the enlargement of existing cells, and the development and medullation of nerve fibers. The *chemical* changes in the growing brain include a decrease in water content, an increase in all lipid constituents, and an increase in protein (12).

The radioactive phosphorus used in this investigation was prepared by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

#### SUMMARY

1. The effects of variation in the amount of phosphorus administered and in the volume of its solution upon the uptake of labeled phosphorus by tissues of the rat were investigated with radioactive phosphorus as indicator.

2. In animals of the same size, the uptake of  $P^{32}$  by blood and brain remained constant irrespective of the volume of solution or the amount of phosphorus injected.

3. The uptake of radioactive phosphorus per *whole* liver, kidney, skeletal muscle, and blood remained constant or increased somewhat as the rat grew larger, whereas in the case of the brain the uptake was markedly reduced.

4. Evidence is presented for the view that recoveries per whole organ provide a more satisfactory basis than recovery per gm. for the comparison of phosphorus and phospholipid activities of the same tissue in animals of different size.

The phosphorus and phospholipid recoveries in liver, kidney, muscle, and brain showed but little difference in the adult mouse and adult rat when comparisons were made on the basis of whole organ.

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# THE PHOSPHORUS METABOLISM OF THE BRAIN AS MEASURED WITH RADIOACTIVE PHOSPHORUS

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It has been observed with the aid of several labeling agents<sup>1</sup> such as claidic acid, deuterium, and radioactive phosphorus (1-4), that the turnover of phospholipids proceeds very slowly in the brain. This slowness is characteristic of all the subdivisions examined; namely, forebrain, cerebellum, medulla, and spinal cord (3). The four divisions, however, do not show a uniform phospholipid activity. Thus, from birth until the time the rat attains a weight of 50 gm., the cord is the most active part of the central nervous system and is 2 or more times as active as the forebrain; the activities of medulla and cerebellum at this time lie between those of cord and forebrain (3). In the older rats (100 to 300 gm.) a change in the relative activities of these divisions occurs; at this time the phospholipid activities of cerebellum, medulla, and forebrain surpass that of the cord in the order given (3).

It has been known for some time that phosphorus penetrates nervous tissue very slowly (4-7). In view of the differences observed in the phospholipid activities of the various brain divisions (3), it seemed desirable at this time to determine whether the pattern noted in these activities is the same for their uptake of total  $P^{32}$ .

## EXPERIMENTAL

The data recorded here were obtained from over 75 rats. Rats of the following weights were used: 5 gm. (new born), 15 gm. (1 week old), 25 gm. (2 weeks old), 30, 50, 100, 200, and 300 gm.

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The manner in which the brain was removed and divided has been described elsewhere (3). In the 5 gm. rat the cerebellum and medulla were combined.

Radioactive phosphorus was administered subcutaneously as an isotonic solution of  $\text{Na}_2\text{HPO}_4$ ; each rat received 1 cc. of this labeled phosphate solution per 100 gm. of body weight. The animals were killed 24 and 48 hours after the injection of phosphorus. Only four weight groups (15, 50, 100, and 300 gm.) were investigated at the 48 hour interval.

The method by which the  $\text{P}^{32}$  content of the brain was determined has been described in the preceding paper (8).

### Results

The total phosphorus activity of the forebrain, spinal cord, cerebellum, and medulla found 24 hours after the administration of radioactive phosphorus is shown in Fig. 1. The results are expressed as *percentage of recovery of the administered labeled phosphorus per whole brain division*. The reason for expressing the results in this manner rather than per gm. of tissue has been given in the preceding paper (8).

The highest phosphorus activity in all four brain divisions was found in the new born rat (5 gm.). During the interval between birth and the time the rat attains a weight of 50 gm. a rapid decline in phosphorus activity occurs. This decline levels off in the 50 gm. rat, and the fall in activity beyond this age occurs very slowly. Thus the forebrain of the 50 gm. rat has an activity only 17 per cent of that found in the 5 gm. rat, whereas the 300 gm. rat has an activity only 5 per cent of that observed in the 5 gm. animal. Similarly the spinal cord of the 50 and 300 gm. rats showed activities of 27 and 9 per cent respectively of those observed in the 5 gm. rat. The cerebellum and medulla were combined in the 5 gm. rat; as a result, the earliest age these tissues were studied separately was 1 week (15 gm. rat). In both these divisions the highest activity was observed in the 1 week-old rat.

Fig. 1 also shows that the rate of decline in phosphorus activity that occurred between the youngest and oldest rats examined was the same for all four divisions.

The total phosphorus activity at the 48 hour interval was 1.25 to 2 times as great as that observed at the 24 hour interval. This was true of all divisions of the brain.

*Comparative Total Phosphorus Activity of Forebrain, Spinal Cord, Cerebellum, and Medulla*—In order to compare the relative activities of these four divisions of the central nervous system, the total phosphorus activity *per gm. of tissue* has been considered. In Fig. 2 the spinal cord has been taken as the standard and its activity in each weight group assigned an arbitrary value of 100.

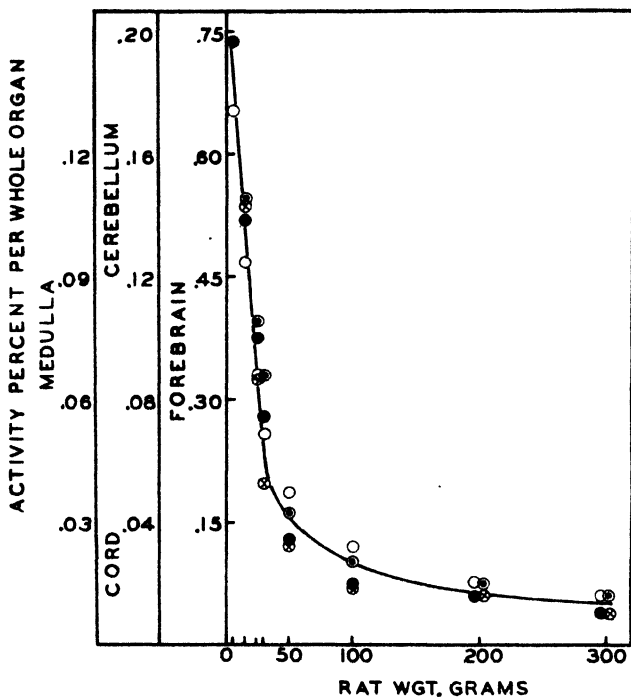


FIG. 1. The total phosphorus activity of whole forebrain, cerebellum, medulla, and spinal cord obtained at the 24 hour interval after the administration of radioactive phosphorus. ○ cord, ⊙ medulla, ⊗ cerebellum, ● forebrain.

The activities of the other divisions are shown in relation to this arbitrary value.

In the youngest animal in which all parts were compared, namely the 15 gm. rat, the activity of the spinal cord was equal to, or slightly lower than, that of the cerebellum; at this time the fore-

brain and medulla showed activities considerably lower than that of the cord. In the 25, 30, and 50 gm. rat the phosphorus activity of the cord was only slightly greater than that of the cerebellum and medulla, but nevertheless appreciably greater than that of the forebrain. In the 200 and 300 gm. rat a change in these relative activities has occurred: the activities of cerebellum and medulla now exceed that of the cord. Thus, from birth until the time the rat reaches a weight of 50 gm., the highest phosphorus activities

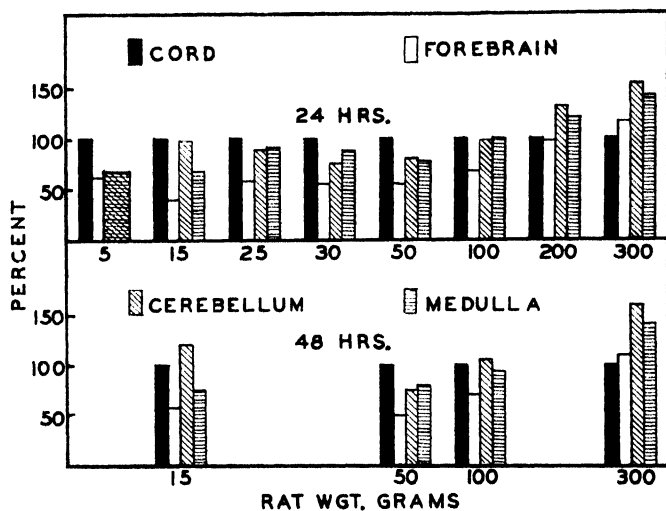


FIG. 2. The comparative total phosphorus activities per gm. of forebrain, cerebellum, medulla, and spinal cord from birth until the time the rat attains a weight of 300 gm. In each group the spinal cord has been assigned the arbitrary value of 100.

were found in either cord or cerebellum, whereas in the 200 and 300 gm. rat the activities in the medulla and cerebellum exceeded those in other divisions.

#### DISCUSSION

In order to correlate the above data on the deposition of labeled phosphorus in the various divisions of the central nervous system with their phospholipid activities, it is desirable to express both sets of results on the same unit basis. In a previous communication (3), the phospholipid activities of the various divisions of the

central nervous system of rats of different ages were expressed *per gm.* of tissue. These phospholipid activities have now been recalculated *per whole brain division*, and as an example the phos-

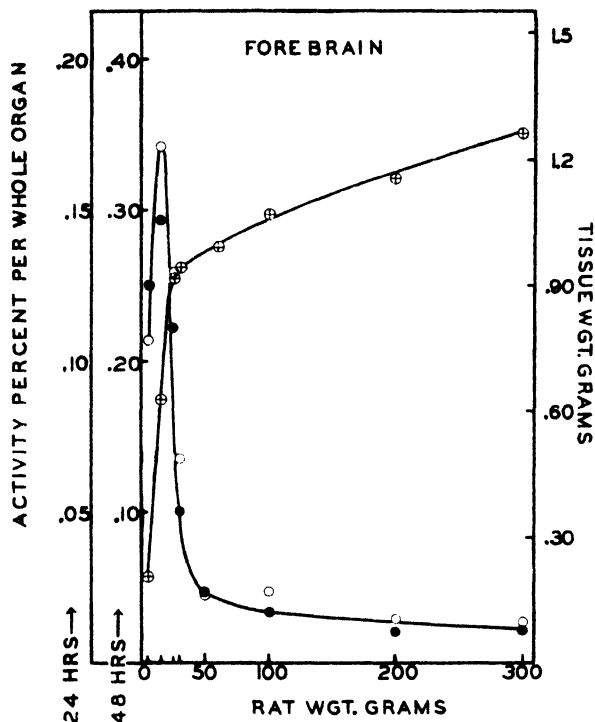


FIG. 3. The phospholipid activity of the forebrain. The ordinates on the left refer to per cent of labeled phosphorus found as phospholipid *per whole* forebrain at intervals of 24 and 48 hours after the administration of radioactive phosphorus. ○ represents average values obtained at the 24 hour interval, ● those obtained at the 48 hour interval. The growth of the forebrain is represented by the symbol ⊕, and the ordinates for these values are shown on the right.

pholipid activities of the whole forebrain<sup>1</sup> in different sized rats are shown in Fig. 3.

<sup>1</sup> The phospholipid recovery *per whole* cerebellum, medulla, and spinal cord can be recalculated from the curves previously recorded (3) by multiplying the phospholipid recovery *per gm.* by the weight of the brain division at each age.

Although it was stated that *per gm.* of tissue the highest phospholipid recovery for each brain division is found in the new born rat, recalculation of the data on the basis of *whole brain division* reveals that the maximum phospholipid activity in the case of the forebrain (Fig. 3) is found in the 1 week-old or 15 gm. rat. In the case of the spinal cord, cerebellum, and medulla, the highest activities occurred in the 2 week-old or 25 gm. rat. Following this rise in phospholipid activity during the 1st and 2nd week after birth, there occurs a sharp decline in phospholipid activities of all brain divisions, which continues until the time the rat reaches a weight of 50 gm. Beyond this stage the decline in phospholipid activity proceeds slowly.

In contrast to these phospholipid activities, the present study shows that the highest recoveries of total radioactive phosphorus are found in all four brain divisions of the new born rat. A similarity, however, is observed in the recoveries of labeled phospholipid and total phosphorus in that the decline that follows the maximum value is precipitous until the rat attains a weight of 50 gm. and thereafter the decline in both activities proceeds very slowly.

The interesting correlation between deposition of total phospholipid (9) and phospholipid activities as measured with radio-phosphorus should be noted here. Thus, maximum deposition of total phospholipid in all parts of the brain occurs in rats between the weights of 15 and 30 gm., *i.e.* between the ages of 1 week and a little over 2 weeks; during this same interval, the highest recovery of radiophospholipid was also found.

Since the recovery of phospholipid  $P^{32}$  represents only a small fraction (20 to 30 per cent) of the total  $P^{32}$  recovery, it is perhaps not surprising that the maximum phospholipid activities found in the forebrain of the 15 gm. rat and in the cord, medulla, and cerebellum of the 25 gm. rat are not reflected in their total phosphorus activities. In the brain of the young rat lipid phosphorus represents nearly one-third of its total phosphorus, whereas in the adult rat at least two-thirds of the total phosphorus of the brain is present as phospholipid (10). Despite this increase in the proportion of lipid phosphorus to total phosphorus that occurs with growth, phospholipid recovery as measured with radioactive phosphorus never exceeds 30 per cent of the total  $P^{32}$  recovered.

The radioactive phosphorus used in this investigation was pre-

pared by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

#### SUMMARY

1. The recovery of total radioactive phosphorus in forebrain, cerebellum, medulla, and spinal cord was compared at 24 and 48 hours after the administration of  $P^{32}$ .

2. The highest  $P^{32}$  recovery in all divisions of the central nervous system was found on the day of birth.

3. From birth until the time the rat attains a weight of 50 gm., a rapid decline in  $P^{32}$  recovery was observed throughout the brain. As growth proceeds beyond 50 gm., the decline continues, but at a much slower rate.

4. The recovery of  $P^{32}$  is not uniform throughout the central nervous system. From birth until the time the rat attains a weight of 50 gm., it is highest in spinal cord or cerebellum. After this the relative activities of forebrain, cerebellum, and medulla rise steadily, and by the time a weight of 200 or 300 gm. is reached the recoveries in cerebellum, medulla, and forebrain exceed that in the cord.

5. The labeled phosphorus present as *phospholipid* did not account for more than 20 to 30 per cent of the total labeled phosphorus deposited in the brain.

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## A COLORIMETRIC REACTION FOR TESTOSTERONE\*

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Since the discovery of the sex hormones, there has been a great need for suitable rapid chemical methods for the quantitative estimation of these hormones in biological materials. Kober (4) in 1931 was the first to apply a color reaction to the quantitative determination of estrogens. By this technique Kober found that estrogens produced a red color when treated with phenolsulfonic acid and sulfuric acid. Later he (5) found  $\beta$ -naphthol in concentrated sulfuric acid to be superior to the original phenol reagent. Other workers (1-3) have studied the reaction as applied to estrogens and have made various modifications.

It was while studying the Kober reaction that Szego and Samuels (6) discovered the value of guaiacolsulfonic acid as a reagent for estrone. Their technique was essentially that of Kober; namely, the development of color with concentrated sulfuric acid and a solution of potassium guaiacolsulfonate, commercially known as thiocol. When testosterone was tested with this thiocol reagent, a blue fluorescence was given. When copper or iron salts were present, a bright green color developed. The production of this bright green color by the action of thiocol reagent on testosterone offered a possible basis for a quantitative method specific for testosterone. Heretofore, the Zimmermann (7) reaction has been the only colorimetric method for androgens, and

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it is not specific, since it is primarily a ketone reagent. Since the thiocol reagent seemed specific for testosterone, it was decided to study the reaction further.

#### EXPERIMENTAL

The specific directions for carrying out the reaction are as follows: A 10 ml. graduated test-tube containing the testosterone (either dry or in about 0.4 ml. of 95 per cent alcohol) is placed in an ice bath. 2 ml. of concentrated sulfuric acid are added and mixed with a footed stirring rod, care being taken to avoid spattering. The tube is heated for 2 minutes in boiling water without stirring, and then placed in an ice water bath. After 5 minutes. 2 ml. of saturated aqueous thiocol and 0.3 ml. of 1 per cent aqueous copper sulfate are added with stirring. The tubes are reheated in boiling water for 2 minutes. During this period they are stirred three times. The tubes are again placed in the ice water bath and diluted to the 10 ml. mark with 50 per cent sulfuric acid. After being transferred to a colorimeter tube, the solution is read against a blank containing the reagents, but not the hormone, in an Evelyn colorimeter equipped with a 635  $m\mu$  filter.

The production of the green compound apparently involves an oxidation. Some green color is produced if the solution without copper or iron salts is vigorously stirred in the boiling water bath. Copper sulfate is used to accelerate this reaction, because it produces the most consistent results. When ferric chloride is used, the green color tends to fade when a small excess is added. A relatively large excess of copper sulfate can be added without any fading. Stronger oxidizing agents such as hydrogen peroxide and potassium permanganate destroy the green color.

Better results are obtained when the thiocol is recrystallized from 60 per cent alcohol. Since thiocol is known to consist of two isomers, recrystallization further concentrates the predominating isomer, which is presumably the one that is responsible for the production of the color.

The calibration curve in Fig. 1 shows the amount of light that is absorbed by various quantities of testosterone with the 635  $m\mu$  filter. Each point on the curve represents the average of many readings. Those points which are off the curve indicate the degree of variation of the readings from the mean. For the smaller

amounts of testosterone the values vary only one or two points from the mean. For 40  $\gamma$  of testosterone the greatest variation from the mean is three or four points. The majority of the values of course fell on or very close to the curve. It seems that from 40 to 80  $\gamma$  the curve veers from Beer's law, and the extent of

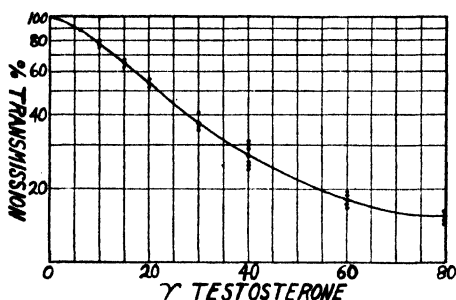


FIG. 1. Calibration curve

TABLE I

*Consistency of Colorimeter Readings for Simultaneous Determinations*

Testosterone	Colorimetric readings			
	Determination 1	Determination 2	Determination 3	Determination 4
$\gamma$				
10	77 79	76 77	79 77 77	
20	55 56	53 53	54 53 54	
30	36 38	35 37 39	36 37 36	
40	27 28 27			27 27

variation from the mean is about the same as for 40  $\gamma$ . Table I shows the colorimeter readings taken for several simultaneous determinations of various amounts of testosterone during 1 day. During a single series of determinations the readings seem to be quite consistent and remain quite consistent throughout the day;

however, from day to day the readings tend to vary more or less. As was indicated in Fig. 1, the variation is never more than three to four divisions on the colorimeter when the reaction is run with 40  $\gamma$  of testosterone and is proportionate to the amount present. For this reason it seems best to run a standard of this strength along with the unknown. For accurate work the amounts in the unknown should then be determined from a curve based on the reading of the simultaneous standard. The curve of Fig. 1 was drawn from the average of a large number of values taken over a period of weeks.

Out of fifteen compounds related to testosterone tested, only three compounds give a green color: testosterone oxime, tes-

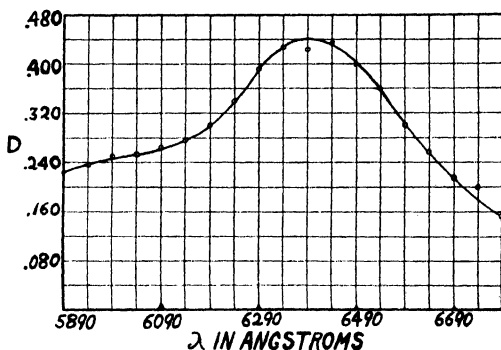


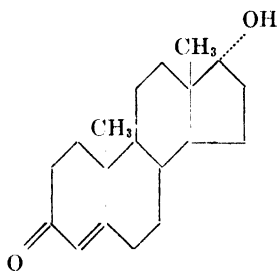
FIG. 2. Absorption spectral curve of the colored compound

tosterone propionate, and androstenedione. The first two are probably hydrolyzed in the reaction medium.

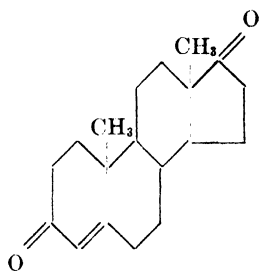
Absorption spectral data indicate that the color produced by androstenedione is identical with that produced by equimolar concentrations of testosterone. The absorption curves for both substances are the same. Fig. 2 shows the absorption curve given by the testosterone reaction. The maximum absorption is at about 6390  $\text{\AA}$ . This absorption curve was obtained by plotting  $D$  versus wave-length.  $D$  is equal to  $-\log T$ .  $T$ , or transmission, is the ratio,  $I/I_0$ , where  $I_0$  is the incident intensity or the intensity of light passing through the blank, and  $I$  is the transmitted intensity or the intensity of light passing through the colored solution. The values of  $I$  and  $I_0$  were determined by means of a

photoelectric spectrophotometer. The colored compound is insoluble in organic solvents except acetone.

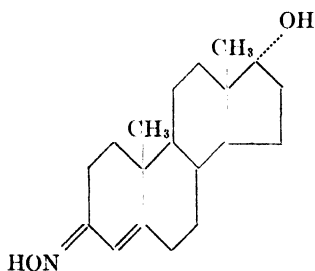
The following compounds have been tested and have been found to give negative reactions by the above procedure: ethynyl-testosterone, androsterone, dehydroandrosterone,  $\Delta^5$ -androstenediol-3-*trans*-17-*cis*, androstanedione-3,17, 3,11,17-androstane-trione,  $\Delta^4$ -3,11,17-androstenetrione, 11-dehydro-17-hydroxycorticosterone, pregnenin-17-diol-3,17, etioallocholanol-3( $\beta$ )-17-one, progesterone, cholesterol, estradiol, estrone, and estriol. By inspecting the formulas of the compounds that do give the test, a very close relationship of the compounds to testosterone can be seen.



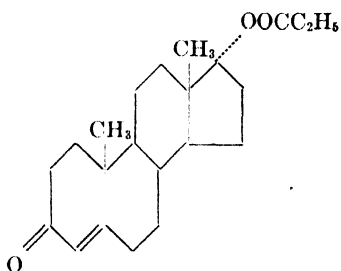
Testosterone



Androstenedione



Testosterone oxime



Testosterone propionate

When testosterone was determined in mixtures of androsterone and dehydroandrosterone, neither androsterone nor dehydroandrosterone interfered appreciably with the production of the testosterone color. Table II gives the results obtained when the mixtures were tested.

A study of the recovery of testosterone from tissues and tissue extracts is in progress.

The thiocol was kindly furnished by Hoffmann-La Roche, Inc. The authors are indebted to Dr. H. L. Mason, Dr. R. I. Dorfman, Dr. F. C. Koch, and Dr. R. D. Shaner for supplying various sterols for testing. In obtaining the absorption spectral curves for testosterone and androstenedione the authors are indebted

TABLE II  
*Behavior of Testosterone Reaction in Presence of Androsterone  
and Dehydroandrosterone*

40 $\gamma$ testosterone with		Trans- mission	Testos- terone found
		per cent	$\gamma$
		27	40
10 $\gamma$ dehydroandrosterone.....		26	42
20 " ".....		27	40
30 " ".....		25	44
40 " ".....		23	48
10 " androsterone.....		25	44
20 " ".....		25	44
30 " ".....		26	42
40 " ".....		26	42
40 " " + 10 $\gamma$ dehydroandrosterone.....		28	39
40 " " + 20 " ".....		25	44
40 " " + 30 " ".....		26	42
40 " " + 40 " ".....		26	42

to Dr. E. S. Miller for the use of the photoelectric spectrophotometer.

#### SUMMARY

A color reaction has been developed for testosterone. Of the eighteen compounds besides testosterone tested, only  $\Delta^4$ -androstenedione-3,17, testosterone propionate, and testosterone oxime give the reaction.

From absorption spectral studies of the colored compound, it has been found that its maximum absorption is at about 6390 Å.

Androsterone and dehydroandrosterone do not interfere appreciably with the testosterone color when mixtures are studied.

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# DETERMINATION OF CHLORIDES IN BIOLOGICAL FLUIDS BY THE USE OF ADSORPTION INDICATORS

## THE USE OF EOSIN FOR THE VOLUMETRIC MICRODETERMI- NATION OF CHLORIDES IN ACETONE FILTRATES OF BIOLOGICAL FLUIDS

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Although both Fajans and Wolff (1) and Kolthoff and his co-workers (2, 3) have pointed out that eosin is an excellent indicator for the titration of bromides, iodides, and thiocyanate in acid solution (particularly in acetic acid medium), Kolthoff (4) has also stated that eosin is so strongly adsorbed in the presence of AgCl, even in the presence of chloride ion, that it cannot be used in the titration of chloride ion.<sup>1</sup> Yet a large number of other investigators (6-10) have published data which prove that under suitable conditions, *e.g.* organic solvents, acid solutions, etc., eosin *can* be used as an adsorption indicator for the titration of chloride ion with silver nitrate even in fairly strong acid media (pH 0.5 to 2.0). One of the most interesting of these papers is that of Burg (10), who points out that accurate end-points can be obtained, with eosin as an indicator, if an organic solvent such as methyl alcohol is present and if the solution is weakly acidic.

Because of the somewhat contradictory evidence which has appeared in the literature on this subject, the authors have investigated the problem of the use of eosin as an adsorption in-

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<sup>1</sup> See also Fajans (5).



indicator in argentometric titrations. It was found that good results could be obtained if the titrations were performed in organic media. For the authors' purpose, *i.e.* microtitrations of chlorides in biological fluids, acetone proved to be the best substance, both because of its ability to precipitate proteins readily to give a water-clear extract and because of the extremely sharp end-point it gave with the indicator. It was also found that unlike dichlorofluorescein (11), eosin in acetone solution was not readily affected by strong oxidizing agents such as hydrogen peroxide. This experimental fact was found to be especially useful for the determination of whole blood chlorides.

With the exception of the paper by Rose (9), who uses a mixed eosin-dichlorofluorescein indicator for macrotitrations of chlorides in urine, no mention has been found in the literature of the use of eosin as an adsorption indicator in the titration of chlorides in biological fluids.

This paper gives the conditions under which rapid, accurate microtitrations of chlorides may be performed in acetone filtrates of various biological fluids when dilute solutions of  $\text{AgNO}_3$  are used as the titrating medium and eosin as the adsorption indicator.

#### EXPERIMENTAL

##### *Solutions—*

Standard  $\text{NaCl}$ , 0.1 N solution. 0.01 and 0.02 N solutions of  $\text{NaCl}$  were prepared by dilution of the 0.1 N solution.

Standard  $\text{AgNO}_3$ , 0.1 N solution. 0.01 and 0.02 N solutions of  $\text{AgNO}_3$  were prepared by dilution of the 0.1 N  $\text{AgNO}_3$ . These dilute solutions were standardized against the standard sodium chloride as follows: 1.0 ml. of 0.02 N  $\text{NaCl}$  was pipetted into a test-tube of convenient size ( $6 \times \frac{3}{4}$  inch). 0.5 ml. of 10 per cent  $\text{H}_2\text{O}_2$ , 7.0 ml. of acetone, and 2 drops of eosin indicator were added. The samples, run in triplicate, were titrated with 0.01 or 0.02 N  $\text{AgNO}_3$  with a 5.0 ml. micro burette calibrated in 0.01 ml. divisions. At the end-point the solution changed from a light pink to a dark purplish pink or mauve color.

Hydrogen peroxide, 10 per cent solution. Diluted from 30 per cent  $\text{H}_2\text{O}_2$  or superoxol (chloride-free).

Acetone, reagent grade (chloride-free).

Eosin indicator, 0.02 per cent in 50 per cent alcohol.

Acetic acid, 50 per cent water solution.

*Analysis of Whole Blood Chlorides*—Pipette 0.2 ml. of whole blood from a standardized 0.2 ml. micro pipette (calibrated to contain) into a 15 ml. centrifuge tube. Rinse the pipette free of cells with two 0.2 ml. portions of distilled water and two 0.2 ml. portions of 10 per cent  $\text{H}_2\text{O}_2$ . Transfer the washings to the centrifuge tube. Dissolve the red blood cells by gently shaking the

TABLE I  
*Determination of Chlorides in Whole Blood (0.2 Ml. Samples)*

Sample No.	Adsorption indicator method	Recovery of added NaCl by adsorption indicator method		Eisenman method	Error
		Added	Recovery		
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent
1	474.0			478.0	
	474.0			476.0	
	474.0			476.0	
	(474.0)			(477.0)	-0.63
2	462.0			467.0	
	456.0			470.0	
	462.0			467.0	
	(460.0)			(468.0)	-1.7
3	480.0			484.0	
	485.0			484.0	
	485.0			478.0	
	(483.0)			(482.0)	+0.21
4	448.0	585.0	590.0		
	448.0	585.0	585.0		
	448.0	585.0	590.0		
	(448.0)	(585.0)	(588.0)		+0.51
5	454.0	585.0	580.0		
	454.0	585.0	580.0		
	448.0	585.0	585.0		
	(452.0)	(585.0)	(582.0)		-0.52

The figures in parentheses are averages for the groups.

tube and add 7.0 ml. of acetone immediately. Stopper the tube with a clean rubber stopper and shake vigorously. Remove the stopper and centrifuge the tube at high speed (above 2000 R.P.M.) for about 5 minutes. Pour off the clear supernatant fluid into a large test-tube ( $6 \times \frac{3}{4}$  inch) which had been previously rinsed with acetone. Rinse the surface of the tightly packed precipitate with two 0.5 ml. portions of acetone and transfer to the large test-tube. Add 2 drops of the eosin indicator and titrate with the

0.02 N AgNO<sub>3</sub> to the first definite mauve coloration appearing throughout the solution.

TABLE II

*Chloride Determinations in Blood Serum or Plasma (0.2 Ml. Samples)*

Sample No.	Adsorption indicator method	Recovery of added NaCl by adsorption indicator method		Van Slyke digestion method	Error
		Added	Recovery		
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent
1. Serum	612.0			617.0	
	612.0			617.0	
	612.0			614.0	
	(612.0)			(616.0)	-0.65
2. Serum	629.0			635.0	
	629.0			620.0	
	626.0			630.0	
	(628.0)			(628.0)	±0.0
3. Serum	597.0	585.0	585.0		
	592.0	585.0	592.0		
	592.0	585.0	580.0		
	(594.0)	(585.0)	(586.0)		+0.17
4. Serum	574.0	585.0	585.0		
	574.0	585.0	585.0		
	567.0	585.0	580.0		
	(572.0)	(585.0)	(583.0)		-0.34
1. Plasma	650.0			650.0	
	642.0			648.0	
	645.0			648.0	
	(646.0)			(649.0)	-0.46
2. Plasma	620.0			628.0	
	620.0			625.0	
	625.0			625.0	
	(622.0)			(626.0)	-0.64
3. Plasma	467.0	585.0	585.0		
	463.0	585.0	590.0		
	474.0	585.0	580.0		
	(468.0)	(585.0)	(585.0)		±0.0
4. Plasma	580.0	585.0	580.0		
	580.0	585.0	590.0		
	585.0	585.0	585.0		
	(582.0)	(585.0)	(585.0)		±0.0

The figures in parentheses are averages for the groups.

Recoveries were determined in the same manner as above after known amounts of standard NaCl solution were added to the whole

blood samples. The results were checked against those obtained with Eisenman's method (12) with 1.0 ml. whole blood samples. A typical set of results is given in Table I.

*Analysis of Blood Serum, Plasma, Cerebrospinal Fluid, or Pleural Fluid*—Pipette 0.2 ml. of serum, plasma, cerebrospinal fluid, or pleural fluid into a 15 ml. centrifuge tube by means of a calibrated,

TABLE III  
*Chloride Determinations in Cerebrospinal Fluid or Pleural Fluid*  
(0.2 Ml. Samples)

Sample No.	Adsorption indicator method	Recovery of added NaCl by adsorption indicator method		Caldwell- Moyer method	Error
		Added	Recovery		
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent
1. Spinal fluid	700.0			709.0	
	700.0			699.0	
	696.0			699.0	
	(699.0)			(702.0)	-0.43
2. Spinal fluid	647.0			655.0	
	655.0			651.0	
	653.0			651.0	
	(652.0)			(652.0)	±0.0
3. Spinal fluid	778.0	585.0	590.0		
	783.0	585.0	585.0		
	790.0	585.0	580.0		
	(784.0)	(585.0)	(585.0)		±0.0
4. Spinal fluid	765.0	585.0	590.0		
	765.0	585.0	590.0		
	760.0	585.0	580.0		
	(763.0)	(585.0)	(587.0)		+0.34
1. Pleural fluid	590.0			588.0	
	590.0			588.0	
	596.0			580.0	
	(592.0)			(585.0)	+1.2

The figures in parentheses are averages for the groups.

mark to mark type, Ostwald-Folin pipette. Add 0.5 ml. of distilled water, 0.5 ml. of 10 per cent  $H_2O_2$ , and 7.0 ml. of acetone. Stopper the tube with a rubber stopper and shake well. The rest of the procedure is exactly as given for the whole blood determinations.

Recoveries were determined in the same manner as above after

known amounts of standard sodium chloride solution were added to the serum or plasma. The results for serum and plasma were checked against those obtained with the Van Slyke (13) open Carius modification of the Volhard method with 1.0 ml. samples for the nitric acid digestion. The results of the runs are given in Table II. The results for spinal fluid and pleural fluid were checked against those obtained by the Caldwell-Moyer (14) modification of the Volhard procedure in which nitrobenzene is used to remove the precipitated silver chloride and sharpen the end-point. A set of recoveries was run for the cerebrospinal fluids. The results of these analyses are shown in Table III.

#### DISCUSSION

The solutions of  $\text{AgNO}_3$  used in these runs were checked against the standard  $\text{NaCl}$  by the well known Volhard procedure as well as by the method of Saifer and Kornblum (15) in which dichloro-fluorescein is used as the adsorption indicator in alcohol-ether media. Both of these methods gave results which checked closely with those obtained by the eosin-acetone method.

The fact that large amounts of  $\text{H}_2\text{O}_2$  have no effect on the end-point of the eosin-acetone method was used to advantage in the whole blood method, the  $\text{H}_2\text{O}_2$  being used to break up and dissolve all the chlorides out of the red cells. It was also found experimentally that 0.5 ml. of 50 per cent acetic acid in an approximate volume of 9.0 ml. served to sharpen the end-point considerably (except for whole blood), the color change being from colorless to purplish pink or mauve rather than the usual change of very light pink to mauve. Although acetic acid could be used for this purpose with plasma, serum, cerebrospinal fluid, or pleural fluid, it did *not* give good results with whole blood titrations.

In checking the adsorption indicator method against well known procedures, such as the Eisenman, Van Slyke, or the Caldwell-Moyer procedure, blanks and standards were run to determine whether the reagents contained noticeable amounts of chloride. Blank corrections were made whenever necessary.

The method as described is rapid, precise, and gives excellent recoveries of added  $\text{NaCl}$ . The results given in Tables I to III for various biological fluids show an average error of less than  $\pm 1$  per cent and a maximum error of less than  $\pm 2$  per cent.

## SUMMARY

A rapid, precise method is presented for argentometric micro-titrations of chlorides in acetone filtrates of various biological fluids with eosin as an adsorption indicator. The method may be used in acid solution, *e.g.* acetic acid, above pH 1 or in the presence of oxidizing agents, *e.g.*  $\text{H}_2\text{O}_2$ , and gives excellent recoveries of added NaCl.

Chlorides in whole blood, plasma, serum, cerebrospinal fluid, and pleural fluid have been determined with an average error of less than  $\pm 1$  per cent.

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## COENZYMES FOR GLYOXALASE

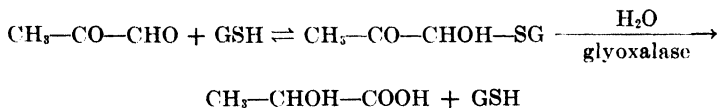
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In 1932 Lohmann (1) first demonstrated the rôle of glutathione as the coenzyme for glyoxalase in the conversion of methylglyoxal to lactic acid. Lohmann, moreover, demonstrated that reduced glutathione was extremely specific in its rôle as coenzyme. Oxidized glutathione, cysteine, thioglycolic acid, hydrogen sulfide, hydrocyanic acid, hydroxyquinoline, citrate, and pyrophosphate were all found to be inactive. To the list of inactive substances Woodward (2) added thioneine and ascorbic acid.

On the basis of iodometric titrations Kühnau (3) postulated the theory that glutathione and methylglyoxal form a combination. Jowett and Quastel (4) presented further evidence indicating that glutathione and methylglyoxal combine reversibly. Their evidence led them to suggest that the addition compound served as the substrate for glyoxalase according to the following equation.



Experiments carried out by Platt and Schroeder (5) supported the view that the addition compound is the intermediate in the formation of lactic acid from methylglyoxal as catalyzed by glyoxalase. The simple addition compound was isolated and analyzed by Schubert (6). In a recent study of the kinetics of glyoxalase activity, Petrovicki (7) reported that the addition compound of glutathione and methylglyoxal prepared according to Schubert's directions was only very slowly acted upon by

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glyoxalase and could not be considered to be the intermediate. He concluded on the basis of his studies that the enzyme must itself be a part of any intermediate product. In view of this latter paper, it is evident that we cannot come to any final conclusion in regard to the nature of the intermediate.

The work cited above, for the most part, supported the view that the sulfhydryl group played a necessary rôle in the coenzyme action. It thus seemed likely that any further compounds possessing such action would probably also be sulfhydryl-containing. That the sulfhydryl group was not in itself the only requirement for coenzyme action had already been demonstrated, as cysteine,

TABLE I

*Rate of CO<sub>2</sub> Production in C.mm. per Hour*

Each column represents a series of experiments at the pH noted. The pH values in parentheses were determined with the glass electrode. Each coenzyme was present in M/120 concentration.

Glutathione	176 (7.4)	168 (6.75)	0 (8.7)
Asparthione	118 (7.8)	112 (7.20)	0 (8.6)
Isoglutathione	57 (7.75)	60 (6.90)	0 (8.5)
Glutathione } Asparthione }	144 (6.80)	160 (6.85)	0 (8.6)
Glutathione } Isoglutathione }	120 (6.90)	128 (6.10)	0 (8.55)
Asparthione } Isoglutathione }	96 (7.60)	108 (6.40)	0 (8.6)
Blank	12 (7.5)	7 (6.8)	

thioglycolic acid, hydrogen sulfide, and thioneine were inactive. It was thus of interest to make use of compounds closely related in structure to glutathione to see whether they were capable of acting as coenzymes. The compound, isoglutathione ( $\alpha$ -glutamyl-cysteinylglycine), differing from glutathione only in that the glutamic acid residue is attached through the  $\alpha$ -carboxyl instead of the  $\gamma$ -carboxyl group, was made available through the work of du Vigneaud, Loring, and Miller (8). Asparthione ( $\beta$ -aspartyl-cysteinylglycine), the aspartic acid analogue of glutathione, has recently been made available through synthesis (9). These two compounds were found to be capable of acting as coenzymes for glyoxalase. As is shown by the values of the dissociation constant

obtained by use of the Michaelis and Menten equation (10), glutathione is the most effective of the substances tested ( $K_s = 7.4 \times 10^{-4}$ ), followed by asparthione ( $K_s = 3.7 \times 10^{-3}$ ) and isoglutathione ( $K_s = 1.23 \times 10^{-2}$ ). Lohmann (1) states that the optimum pH for the conversion of methylglyoxal to lactic acid in the presence of glutathione lies between pH 6.5 and 7.5. The pH optimum in the presence of asparthione or isoglutathione proved likewise to be broad and to lie in a similar range. In the presence of approximately optimal amounts of any two of the coenzymes the rate of enzyme action was intermediate between that found with the coenzymes used alone (Table I). This is to be expected if the coenzymes are simply competing reversibly one with another for combination with the enzyme or substrate or with an enzyme-substrate complex. The  $V_{\max}$  for the three substances as indicated by the extrapolated plots are very similar (glutathione 172, asparthione 161, and isoglutathione 143). This likewise provides strong evidence that all three of the substances function in the same manner with no non-competitive effects. In contrast to asparthione and isoglutathione, cysteinylglycine was not effective as a coenzyme.

#### EXPERIMENTAL

The acetone-yeast to be used as the source of glyoxalase was prepared from fresh pressed bakers' yeast (Standard Brands No. 189) according to the procedure of Albert, Buchner, and Rapp (11). The dry preparation thus obtained was washed as suggested by Platt and Schroeder (5) and again dried by the acetone-ether treatment. The dry solid residue was preserved in the ice box, being made up into a 5 per cent suspension for use as needed. Methylglyoxal was prepared by heating acetone with selenium dioxide as described by Riley, Morley, and Friend (12). The methylglyoxal content of the solution obtained was determined as the *m*-nitrobenzoylosazone (13), and the solution was then diluted so as to contain approximately 5 mg. per cc. The glutathione, isoglutathione, and cysteinylglycine used in these experiments were crystalline synthetic products which were generously furnished by Professor Vincent du Vigneaud. The asparthione was likewise a synthetic product synthesized as described by Miller, Behrens, and du Vigneaud (9).

The conversion of methylglyoxal to lactic acid was measured manometrically in Warburg vessels in a manner similar to that described by Lohmann (1). In a side bulb were placed 0.1 cc. of 0.2 N  $\text{NaHCO}_3$  and an aqueous solution containing approximately 1.2 mg. of methylglyoxal. The reaction vessel contained 0.4 cc. of 0.2 N  $\text{NaHCO}_3$ , 0.3 cc. of a 5 per cent suspension of yeast, a solution of glutathione or of the substance being tested for coenzyme activity, and water to bring the total volume to 2.3 cc. The vessels were equilibrated with  $\text{N}_2$  containing 5 per cent  $\text{CO}_2$ .

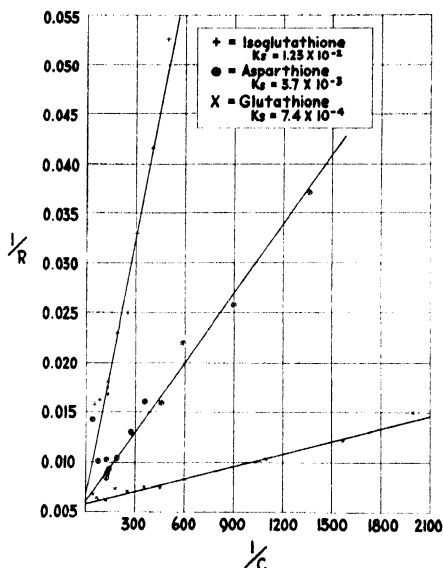


FIG. 1. Evaluation of  $K_s$  and  $V_{\max}$ .  $R$  = initial reaction velocity in c.mm. of  $\text{CO}_2$  produced per hour;  $C$  = molal coenzyme concentration.

at  $25^\circ$ , with shaking, and the contents of the side bulb were tipped into the reaction vessel. Readings were taken every 5 or 10 minutes for several hours. It was observed that the rate became constant within 10 minutes and remained constant for the 1st hour or  $1\frac{1}{2}$  hours, after which time some falling off of rate occurred owing to enzyme destruction. The total production of  $\text{CO}_2$  corresponded closely to that required by the complete conversion of the methylglyoxal into lactic acid. Controls to which no

coenzyme was added produced  $\text{CO}_2$  at a rate of about 12 to 14 c.mm. per hour.

The data were plotted (Fig. 1) in the manner suggested by Lineweaver and Burk (14). These authors have pointed out that when  $1/R$  is plotted against  $1/C$ , the ordinate intercept is  $1/V_{\max.}$  and the slope of the straight line is  $K_s/V_{\max.}$ , thus evaluating  $K_s$ . ( $K_s$  is the dissociation constant for the substance  $s$  and the enzyme. Usually  $s$  refers to the substrate; however, in our case it refers to the coenzyme.  $R$  is the reaction velocity,  $C$  may represent the coenzyme concentration, and  $V_{\max.}$  is a numerical constant representing the maximum velocity obtained when the enzyme  $E$  exists completely in the form  $Es$ .) It will be noted that with increasing higher concentrations of the coenzymes, the rate does not continue to increase as might be expected from the curve, but reaches a maximum and indeed tends to decline, owing presumably to some incidental effect. Platt and Schroeder (5) describe this as "a slight toxic effect."

I wish to express my appreciation to Professor Vincent du Vigneaud, at whose suggestion the problem was undertaken, and to Professor Dean Burk for helpful advice in carrying out this work.

#### SUMMARY

Both isogluthathione ( $\alpha$ -glutamylcysteinylglycine) and asparthione ( $\beta$ -aspartylcysteinylglycine) have been demonstrated to be capable of acting in lieu of glutathione as coenzymes for the action of glyoxalase in transforming methylglyoxal to lactic acid. The pH optimum for the enzyme action proved to be broad and to lie in a similar range for all three substances. Asparthione ( $K_s = 3.7 \times 10^{-3}$ ) was somewhat less effective than glutathione ( $7.4 \times 10^{-4}$ ), and isogluthathione ( $1.23 \times 10^{-2}$ ) was considerably less effective. In the presence of optimal amounts of any two of the coenzymes the rate of enzyme action was intermediate between that found with the coenzymes used alone. The extrapolated maximum velocities were approximately the same with all three coenzymes. These two facts signify that all three of these coenzymes function in a similar manner. Cysteinylglycine was without effect as a coenzyme.

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# EFFECT OF DIET ON GLUCOSE ABSORPTION BY THE RAT

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Although the nature of the mechanism involved seems still to be poorly understood (1), it has long been accepted as a fact that the absorption from the intestine of the common hexoses, glucose, galactose, and fructose, is an active process rather than a passive diffusion. In 1933 MacKay and Bergman (2) suggested that the efficiency of the process of glucose absorption was influenced by the composition of the maintenance diet. Rats fed on a high carbohydrate diet appeared to absorb glucose more rapidly than those fed on a high fat diet. The following year Westenbrink (3) demonstrated quite conclusively that the composition of the previous diet exerts a profound effect on the rate of absorption of glucose, fructose, and galactose by the rat.

From the work of Wesson (4), of Wesson and Burr (5), and of Burr and Beber (6) it appears that rats that are suffering from a deficiency of the essential unsaturated fatty acids have an abnormal carbohydrate metabolism in that, immediately after its ingestion, they convert an excessive amount of sugar into fat. Later this synthesized fat is all burned and the net result is an extremely emaciated rat practically devoid of depot fat.

We were curious to learn to what extent the excessive conversion of glucose into fat might be due to an abnormally rapid absorption of glucose from the intestine. Having on hand a number of rats that had been raised on a high carbohydrate, low fat diet (Diet 3 (7)), we determined the rate of glucose absorption.

It was found that these absorbed about  $1\frac{1}{2}$  times as much glucose as the stock controls. On the other hand, feeding the same high carbohydrate, low fat diet to a group of mature rats

for 3 weeks or more caused no significant change in the rate of glucose absorption. Evidently the stock ration (Purina Fox Chow) was sufficiently rich in carbohydrate so that the elimination of practically all fat from the diet had no effect on the efficiency of the mechanism involved in glucose absorption. If, however, mature rats were changed from the stock ration to one very rich in fat and low in carbohydrate, their capacity to absorb glucose was decidedly diminished. These results suggest that the excessive rate of glucose absorption by the rats that had been raised on the high carbohydrate diet may indeed have been a consequence of the deficiency of the essential unsaturated fatty acids.

#### EXPERIMENTAL

The procedure used to determine the rate of glucose absorption was that developed by Cori (8). After a 48 hour or a 24 hour fast, the rats were given orally a measured volume of a glucose solution. 1 hour later the rats were killed and the unabsorbed glucose was recovered from the gastrointestinal tract by washing with water. The glucose in the wash water was determined by Bertrand's method.

#### *Results*

The essential data obtained on six groups of rats have been summarized and condensed into Table I. The statistics  $t$  and  $P$  have been calculated according to the method of Fisher ((9) p. 114).  $P$  indicates the probability that the rates of glucose absorption by the two groups are identical.

The males in Group A were intended to serve as controls with which to compare the ten males in Group B that had been raised on the high carbohydrate, low fat diet. In the early stages of the work, we were disturbed by the high degree of variability in the rate of glucose absorption. We suspected some fault in technique, and, while we were trying to detect and eliminate it, the number of animals used became larger than had been intended. It presently became apparent that the variation from one day to another was in general greater than from one animal to another on the same day. We are inclined to believe that one important factor involved in the variability among animals was the temperature and

humidity of the room in which the experiments were carried out. On a day of high temperature and humidity the rats were languid and the rate of glucose absorption was likely to be depressed.

When the experiments on the rats in Group B were finally begun, it was at once found that these animals would not stand a 48 hour

TABLE I  
*Effect of Diet on Glucose Absorption by Rat*

Group	Dietary regimen	No. of rats	Final body weight	Glucose per 100 gm. body weight			Difference		<i>t</i>	<i>P</i>
				Fed	Absorbed		From Group	Per 100 gm. body weight		
			gm.	mg.	mg.			mg.		
A*	Purina Fox Chow	25 ♂	257	622	120 ± 48		F	51	3.79	<0.001
F	" "	14 ♀	204	634	171 ± 20					
E	Fasted to 70% of weight	11 ♂	209	454	172 ± 27		F	1		
B	Raised on high carbohydrate, low fat diet	10 ♂	167	752	267 ± 77		"	96	4.48	<0.001
C	High carbohydrate, low fat for 3 wks. or more	12 ♂	249	595	157 ± 45		"	14	1.03	0.3
D	High fat, low carbohydrate for 3 wks. or more	19 ♂	250	394	109 ± 21		"	62	8.35	<0.001
							C	48	4.00	<0.01

\* This group was fasted for 48 hours; all others were fasted for 24 hours.

fast. These rats had already lost some weight and were not in good condition. Accordingly it was felt necessary to shorten the preliminary fasting period to 24 hours. And, in view of the findings of Cori (10) that rats fasted for 24 hours absorb glucose more rapidly than those fasted for 48 hours, it was necessary to set up another group of controls. As sufficient males of a suitable



size were not available, we were forced to use females. Both Cori (8) and MacKay and Bergman (11) have found that the rate of glucose absorption is the same in males and females. Our data indicate that rats fasted for 24 hours (Group F) absorb, on the average, about 40 per cent more glucose in 1 hour than rats fasted for 48 hours (Group A).

It will be seen that the rats in Group B, that had been raised from weaning age on the high carbohydrate, low fat diet, absorbed on the average 96 mg. more glucose per 100 gm. of body weight than the controls in Group F. The difference is clearly significant. Now, the rats in Group B, as a consequence of the prolonged deficiency of the essential unsaturated fatty acids, had already declined somewhat from their maximum weight. In view of the fact that the absorbing capacity of the intestine is presumably a function of its size, we wondered if it were possible that the higher rate of absorption of glucose by the rats in Group B was due mainly to the loss in body weight, assuming that the active absorbing mass of the intestine did not decline in proportion to the loss in weight. We thought to test out this possibility by fasting stock rats until they had lost about 30 per cent of their body weight and then determining their rate of glucose absorption. However, it was found that such rats, after a prolonged fast, could not tolerate the customary dose of glucose. A profuse diarrhea developed, often within 20 minutes, even after the administration of as little as 0.5 cc. of 50 per cent glucose solution. A single feeding of the stock ration restored their tolerance to the extent that 2 cc. of 50 per cent glucose solution could be given without the diarrhea developing. Accordingly, the eleven males in Group E were fasted until they had lost 30 per cent of their initial weight and then were fed for 1 day on the stock ration. They were then fasted for 24 hours and given 2 cc. of 50 per cent glucose solution. It will be seen from Table I that these males absorbed on the average 172 mg. of glucose per 100 gm. of body weight. Their absorption coefficient is the same as that of the controls in Group F. It appears evident therefore that the absorbing capacity of the intestine decreased with the decline in body weight.

MacKay and Bergman (11) have claimed that it is more correct to express the absorptive capacity of the animal in terms of its surface area than its weight. This may well be true for animals of widely different ages and therefore weights; but it does not have

any real advantage for mature animals differing by as much as 100 gm. or more in weight. Group F was actually made up of two subgroups; one ranged from 172 to 191 gm. and averaged 180 gm.; the other ranged from 205 to 304 gm. and averaged 249 gm. The former absorbed an average of  $174 \pm 21$  mg. and the latter  $168 \pm 21$  mg. per 100 gm. of body weight.

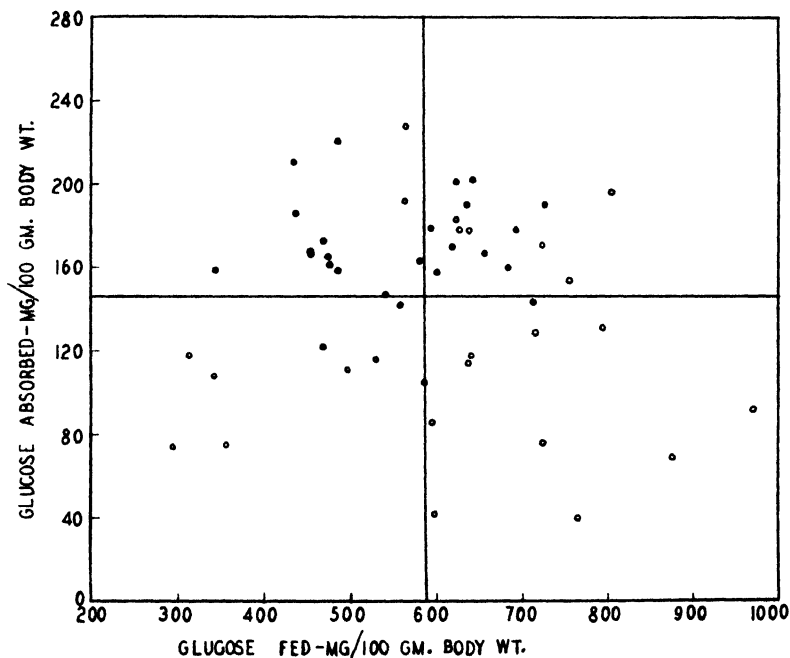


FIG. 1. Showing the lack of correlation between the amount of glucose absorbed in 1 hour and the amount of glucose fed. ● rats in Groups F and E; ○ rats in Group A.

To study further the effect of diet on the rate of glucose absorption, a group of stock males was placed on a high fat diet, and another group on a high carbohydrate diet (Diet 3). The high fat diet consisted of casein 28.1, salt mixture (12) 6.3, yeast 14.4, commercial hydrogenated fat 51.2 per cent. The high carbohydrate diet consisted of casein 17.1, sucrose 70.3, salt mixture 3.8, yeast 8.8 per cent. Both diets were supplemented by 1 drop of percomorph oil daily.

The data in Table I show clearly that the high carbohydrate diet did not increase the rate of glucose absorption above that of the controls (Group F). The high fat diet, on the other hand, decreased the absorptive capacity of the intestine very materially, thus confirming the findings of others (2, 3).

In Groups A, F, and E the amount of glucose fed ranged from 300 to 970 mg. per 100 gm. of rat. Fig. 1 quite clearly confirms the fact first established by Cori (8) that the amount of glucose absorbed bears no relationship to the amount of glucose fed. Fig. 1 also brings out strikingly the wide variability in the absorptive capacity of the intestine from one rat to another within the same group.

#### SUMMARY

Rats which had been raised from weaning age on a high carbohydrate, low fat diet and which were suffering from a deficiency of the essential unsaturated fatty acids absorbed  $1\frac{1}{2}$  times more glucose per 100 gm. of body weight than a control group of stock animals. Mature rats fed for 3 weeks or more on this same high carbohydrate diet did not show any change in their capacity to absorb glucose. Mature rats fed for 3 weeks or more on a high fat diet showed a pronounced decrease in their rate of glucose absorption.

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# THE MODIFICATION OF CUPRIC CHLORIDE CRYSTALLIZATION PATTERNS BY TRACES OF PROTEINS

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## PLATE 1

(Received for publication, August 1, 1941)

In a recent paper on the isolation of glycogen from maize (1), it was stated, "The cupric chloride crystallization pattern of glycogen is very similar to, but not identical with, that of sweet corn extracts. However, the crystallization pattern of the glycogen preparations from sweet corn is indistinguishable from that of glycogen from animal sources." The implication was that something which modifies the crystallization pattern occurs, together with glycogen, in the sweet corn. The nature of this "something" was made clear during attempts to purify the polysaccharide responsible for the crystal pattern obtained with extracts from oats (*cf.* (2)).

In the course of the purification of the oat seed polysaccharide, no significant change occurred in the crystal pattern when the preparation was precipitated from water by alcohol, acetone, or basic lead acetate. The pattern of a crude oat extract, and of preparations so precipitated, is shown in Fig. 1. When the preparation was precipitated from water by the addition of acetic acid, however, the pattern was that of Fig. 2, which has few recognizable points of similarity with the "oat pattern." It seemed possible that a little protein had been carried through the early steps of the purification, but was removed by the acetic acid treatment. Accordingly a trace of egg albumin was added to some of the acid-treated preparation; the resulting pattern, Fig. 3, was indistinguishable from a "normal" oat pattern.

Since the addition of protein to the oat polysaccharide had such a pronounced effect, an attempt was made to see how the glycogen pattern would behave under similar circumstances. None of the usual methods for the purification of glycogen made any funda-

mental change in the pattern, which was of the type shown in Fig. 4. When, however, corn glycogen was subjected to repeated acetylation and saponification, a different pattern was obtained (Fig. 5). As in the case of the oat preparation, the addition of a very little protein (of the order of 0.5 per cent) resulted in the normal glycogen pattern, and of a little more (of the order of 1.5 per cent), the accentuated pattern shown in Fig. 6. The differences between these patterns are more clearly shown by the photomicrographs in Figs. 7 and 8. By adjustment of protein concentration, patterns closely resembling those of crude corn extracts could be obtained.

Liver glycogen could not be brought to the point at which it gave the "pure glycogen" pattern (Fig. 5), even by repeated acetylation and saponification. The pattern was never very different from that of Fig. 4; but addition of 1.5 per cent of protein to it resulted in the heavily branched pattern of Fig. 6. Starch which had been twice acetylated and saponified gave a pattern which differed from the normal in color and texture. The differences observed in this case are not evident in photographs. With protein the normal pattern was restored.

It was found that polyvinyl alcohol, a synthetic polymer, gave with cupric chloride a definite crystal pattern which was also found to be radically affected by the addition of proteins. Figs. 9 and 10 show the result with the pure alcohol, and Figs. 11 and 12 show the effect of the addition of a trace of egg albumin.

In all the cases mentioned, extremely small amounts of proteins give observable effects. The amount required seems to bear no relation to the amount of polysaccharide, but only to the total amount of cupric chloride. 0.5 gm. of cupric chloride is used on each plate, and 0.1 to 0.3 mg. of protein seems to be the most effective amount to add in the case of most of the polysaccharides tried. As little as 0.01 mg. (0.05 per cent) can be detected in the case of glycogen, and as much as 1 mg. (200 per cent in the case of the oat preparation) can be added without entirely obliterating the polysaccharide patterns.

The protein effect is quite non-specific. It is given about equally well by crystalline egg albumin, the water-soluble globulin of oats, crystalline oat globulin, whole blood, tobacco mosaic virus, and certain enzyme preparations.

The question naturally arises as to whether other substances have an effect comparable to that of the proteins. Since proteins markedly affect the surface tension of water, the effect of other surface-active substances was investigated. Organic solvents, such as acetic acid, toluene, or ethyl acetate, have no noticeable effect. The commercial wetting agent, Aerosol (which itself gives a pattern) produced an effect which was obviously a superposition of its own pattern on that of the pure polysaccharide. Commercial sodium taurocholate gave an effect similar to that of the proteins, but only when an excessive amount (3 mg.) was used. This quantity was so large that it suggested the possibility of contamination with adventitious protein. Consequently synthetic sodium taurocholate was used, and was found not to be effective at any concentration. Instead, like Aerosol, it gave a superposition of its own pattern (irregularly placed long crystals) upon that of the polysaccharide (see Fig. 13, with oat polysaccharide).

In contradistinction to these substances, the addition of protein to polysaccharide produces not two superposed patterns, but one integrated one, in which the protein pattern is entirely submerged unless the protein is present in great excess. In the latter case, superposition may be observed, and the polysaccharide pattern may even be obliterated. Proteins alone give a pattern like that of Fig. 14.

#### EXPERIMENTAL

*General Procedure*—The crystallizations were carried out by the methods described previously (2). Solutions to be tested were added to water and stock cupric chloride solution, so that the final concentration of cupric chloride was 0.5 gm. in 8 cc. The polysaccharide and protein solutions were mixed before the addition of the cupric chloride, in order that this latter might not prevent formation of addition compounds (*cf.* (3)). The mixtures were poured on flat bottom dishes, and allowed to evaporate spontaneously at constant temperature (27–29°) in a closed room.

*Preparation of Addenda*—For the oat preparations, ground oat seeds were extracted with water at room temperature. To the filtered and boiled extract were added 2 volumes of alcohol. The precipitate was dissolved in water, and precipitated by the addition of 2 volumes of glacial acetic acid.

The glycogen samples were prepared as described in an earlier paper (1).

Any crude starch may be used, either natural, as a paste, or made "soluble" by being boiled with alcoholic hydrochloric acid (4).

The egg albumin had been recrystallized three times.

Crystalline oat globulin was prepared as described by Osborne (5).

A preparation of oat seed protein which has properties convenient for the present purpose has been prepared as follows (it appears to be somewhat similar in properties to one described by Osborne (6) as Preparation 20): Ground oat seeds were extracted with 5 per cent salt solution, and the filtered extract was dialyzed until it was nearly (but not entirely) salt-free. The precipitated globulins were separated in the centrifuge and washed once with very dilute salt solution. The product was dissolved in 5 per cent salt solution, filtered, and the filtrate was saturated with salt. The white precipitate which separated was washed twice with very dilute salt solution and dissolved to form an opalescent solution in distilled water. To this, 0.2 N acetic acid was added until it became clear (at about pH 5). This was filtered, and 0.2 N ammonia was added until the globulin precipitated, at about pH 6.2. The preparation may be further purified by repeated solution and precipitation near pH 6 with the use of 0.02 N reagents. A solution of this protein in 0.02 N acetic acid seems to keep indefinitely under toluene at room temperature, or the protein may be precipitated with acetone and dried.

*Acetylation and Saponification*—The polysaccharides were acetylated by the method of Haworth, Hirst, and Isherwood (7) with pyridine and acetic anhydride. In each case the crude acetyl compound was dissolved in ethyl acetate, filtered, and precipitated with alcohol. It was usually found necessary, after the second acetylation, to add a little ammonium acetate to the ethyl acetate-alcohol mixture to coagulate the opalescent sol that was formed.

The reprecipitated acetyl derivative was washed with alcohol and saponified by being shaken with 0.5 N alcoholic potassium hydroxide solution for half an hour; after this the alkali was neutralized with acetic acid, and the recovered polysaccharide was dissolved in water and reprecipitated with alcohol.

## SUMMARY

Minute quantities of proteins can greatly modify the cupric chloride crystallization patterns produced by polysaccharides and similar substances. This modification is apparently not due entirely to surface tension effects.

The specificity of the patterns so far described appears to be due to the polysaccharides, whereas the action of the proteins is entirely non-specific.

The protein effect seems to depend upon the total amount of protein present, rather than on the proportion of protein to polysaccharide.

We wish to express our gratitude to the American Association for the Advancement of Science for a grant-in-aid; and for materials, suggestions, or both, to Dr. H. T. Clarke and Dr. F. Cortese of the College of Physicians and Surgeons of Columbia University, Dr. E. A. Hauser of the Massachusetts Institute of Technology, Dr. M. A. Lauffer of The Rockefeller Institute for Medical Research, and Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station.

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## EXPLANATION OF PLATE 1

Cupric chloride crystallization patterns.

FIG. 1. 0.2 cc. of an extract of oats with 5 parts of water.

FIG. 2. 0.5 mg. of the oat polysaccharide precipitated with acetic acid.

FIG. 3. The same, plus 0.1 mg. of egg albumin.

FIG. 4. 20 mg. of glycogen, purified by precipitation with acetic acid.

FIG. 5. 20 mg. of glycogen, twice acetylated and saponified (0.006 per cent nitrogen).

FIG. 6. The same, plus 0.3 mg. of egg albumin.

FIG. 7. Two portions of the plate of Fig. 5.  $\times 4$ .

FIG. 8. A portion of the plate of Fig. 6.  $\times 4$ .

FIG. 9. 10 mg. of polyvinyl alcohol.

FIG. 10. A portion of the plate of Fig. 9.  $\times 4$ .

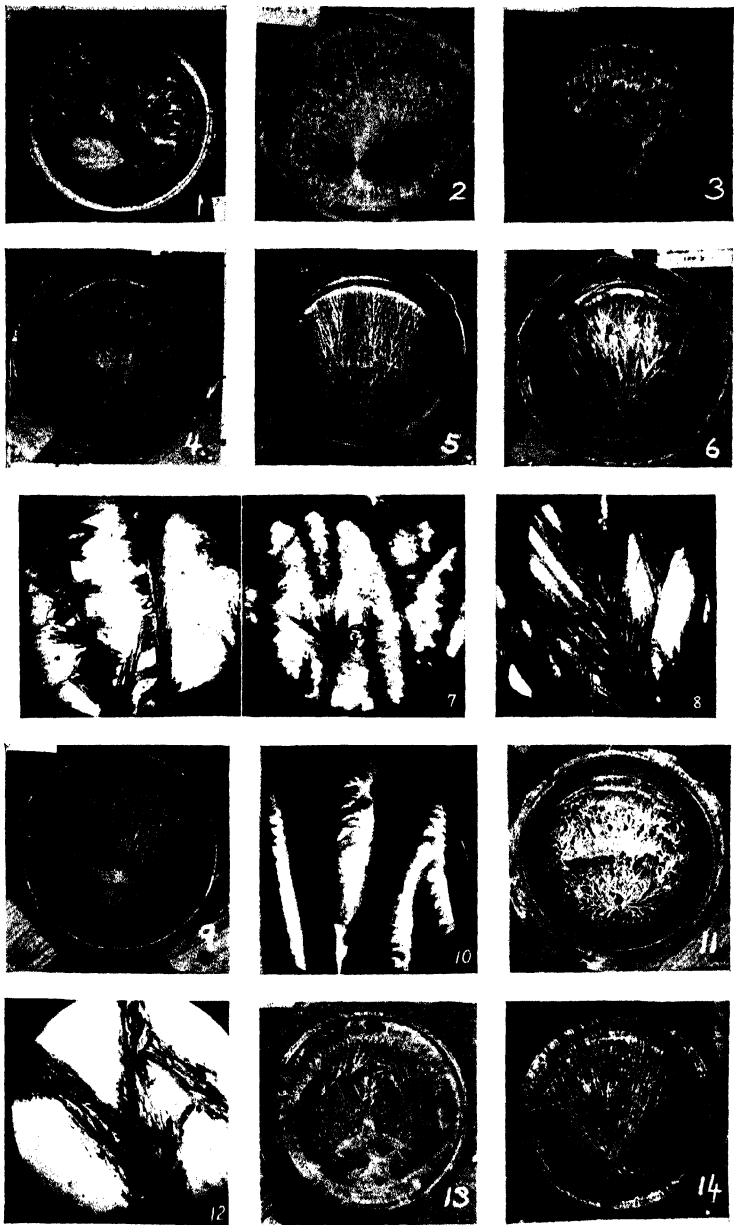
FIG. 11. 10 mg. of polyvinyl alcohol plus 0.1 mg. of egg albumin.

FIG. 12. A portion of the plate of Fig. 11.  $\times 4$ .

FIG. 13. 0.5 mg. of the oat polysaccharide plus 3 mg. of sodium taurocholate (synthetic).

FIG. 14. 0.1 mg. of egg albumin.

Note: Figs. 1, 2, 3, 4, 5, 6, 9, 11, 13, and 14 are about one-fourth actual size.



(Morris and Morris: Proteins and crystal patterns)



## GROWTH FACTORS FOR BACTERIA

### XIII. PURIFICATION AND PROPERTIES OF AN ELUATE FACTOR REQUIRED BY CERTAIN LACTIC ACID BACTERIA\*

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Recent work on the nutrient requirements of *Lactobacillus casei*  $\epsilon$  has shown that the essential growth factors of unknown composition other than those found in hydrolyzed casein may be supplied by yeast extract and can be separated by adsorption on norit A into the filtrate and eluate fractions. The filtrate fraction can be replaced by biotin, thus leaving only the eluate fraction as unknown. Snell and Peterson (1) in preliminary work on this fraction reported that the compound had some acidic properties but basic ones predominated. More recently Stokstad (2) has prepared a concentrate of the factor which because it contained nitrogen, phosphorus, a pentose, and guanine led him to the conclusion that the active compound is a nucleotide. The factor was partially replaceable by thymine and guanine. However, this report does not contain adequate data for establishing the purity of the preparation and its identity with that of the growth factor. That the preparation must have contained much impurity is shown by the note of Mitchell, Snell, and Williams (3) who reported a fraction more than 100 times as active as Stokstad's preparation. Their fraction contained no phosphorus, which excludes the possibility of the factor being a nucleotide. It contained nitrogen and from diffusion experiments appeared to have a molecular weight of about 500. The Texas group believe their preparation is essen-

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tially a pure compound and, since they obtained it from a leafy material, propose to call it "folic" acid. This name appears unwarranted as the factor was first obtained from yeast and is abundant in liver, milk, peptone, and many other natural materials. It would seem more appropriate to defer naming the factor until its chemical identity has become established.

Recently Hutchings *et al.* (4) have presented evidence to show that the fractions which are active for the bacteria also contain a factor required by the chick. Parallel concentration and inactivation strongly suggest that the bacterial and chick factors are identical.

In this paper we wish to present an improved method for the concentration of the factor and additional observations on its nature and biological importance.

#### EXPERIMENTAL

The bacteriological technique and basal medium are the same as those used previously. Similarly, 1 unit of activity is defined as that amount of material which must be added to 10 cc. of medium to produce half maximum fermentation.

The procedure for the preparation of purified concentrates of the norit eluate factor is as follows: 1 kilo of solubilized liver powder<sup>1</sup> is dissolved in 20 liters of water. The solution is adjusted to pH 3.0 with concentrated sulfuric acid, Filter Cel is added, and the flocculent precipitate is filtered off. 200 gm. of norit A are added to the filtrate and stirred for 1 hour. The solution is filtered and the filtrate discarded. The norit is suspended in 2 liters of 50 per cent ethanol, stirred 40 minutes, filtered, and the filtrate discarded. The norit is suspended in 5 liters of 10 per cent ammonia and 50 per cent ethanol and stirred at 70° for 1 hour. This is repeated once. The combined eluates are concentrated to dryness. The material contains 1 unit in 1  $\gamma$  and represents a yield of 60 per cent. It is dissolved in enough water to contain 20 mg. per cc. and the solution adjusted to pH 3.0 with sulfuric acid. The flocculent precipitate which forms is filtered off and superfiltrol

<sup>1</sup> The solubilized liver fraction is that portion of an aqueous liver extract precipitated from solution by addition of ethanol to 70 per cent concentration, then rendered water-soluble by enzyme action. We wish to thank Dr. David Klein, of The Wilson Laboratories, Chicago, for this preparation.

equivalent to twice the weight of the solids in the filtrate is added. The suspension is stirred 1 hour, filtered, and the filtrate discarded. The superfiltrol cake is suspended in 4 liters of the ammonia-ethanol mixture and eluted twice at room temperature for 1 hour each time. The eluates are combined and concentrated to dryness. The material has an activity of 1 unit in 0.5  $\gamma$  and represents a 70 per cent yield of the previous fraction. It is dissolved in water and again adsorbed on norit A and eluted with the ammonia-ethanol mixture. On a dry basis this eluate has 1 unit in from 0.09 to 0.1  $\gamma$  and contains about 60 per cent of the activity of the preceding fraction. The over-all yield is 24 per cent and the factor has been concentrated 180 to 200 times.

#### *Miscellaneous Properties of Norit Eluate Factor*

*Acid and Alkali Stability*—Material having an activity of 1 unit in 0.4  $\gamma$  loses 75 per cent of its potency on heating with 1  $N$   $H_2SO_4$  at 125° for 15 minutes. With 1  $N$   $NaOH$  under similar conditions only 25 per cent of the activity is destroyed. With 1  $N$   $H_2SO_4$  at 100° almost 30 per cent of the activity is lost in 4 minutes. After the initial large loss of activity there is just a steady decrease as shown in Table I.

Since the factor is acidic in nature it was thought that acid lability might be due to lactone formation. However, the inactivated material could not be regenerated by treatment with sodium hydroxide and hence this change appears to be excluded.

*Precipitation with Heavy Metals*—With material containing 1 unit of activity in 0.4  $\gamma$  and in 5 per cent concentration, the factor was completely precipitated by mercury, zinc, and copper, from 50 to 75 per cent by silver, cadmium, and barium, and about 20 per cent by calcium and nickel. Zinc is the only metal offering enough selectivity to be of aid in purification and has been used in certain purification operations. Barium-EtOH precipitations have not yielded clear cut separations.

*Precipitation with Certain Specific Reagents*—The factor is not precipitated by ammonium rhodanilate, ammonium reineckate, flavianic acid, picrolonic acid, or picric acid. Since these are well known basic precipitants, this is some evidence that the factor is less basic than was previously thought.

*Adsorbents*—The factor is much more strongly adsorbed at acid

than at neutral or alkaline pH. For this reason all adsorptions have been carried out at pH 3.0. The factor is quantitatively adsorbed on norit, fullers' earth, superfiltrol, and to a lesser degree on aluminum hydroxide C $\gamma$ , aluminum oxide, and anthranilic acid. With the last three substances, either because the factor is too tenaciously held or is destroyed in the process of adsorption, it has been possible to elute only a small portion of the original activity.

*Solubility*—The factor is insoluble in all the common organic solvents with the exception of glacial acetic acid, formamide, and dioxane (only slightly soluble).

*Treatment with Enzymes*—The norit eluate factor is not destroyed by pepsin, trypsin, ficin, yeast peptidase, or a commercial preparation of phosphatases. Of interest is the fact that the factor is

TABLE I  
*Acid Lability of Eluate Factor*

Heating in 1 N H <sub>2</sub> SO <sub>4</sub> at 100°	Weight having 1 unit activity	Destruction
<i>min.</i>	$\gamma$	<i>per cent</i>
0	0.21	
4	0.30	29.8
8	0.325	35.3
16	0.36	41.6
32	0.38	44.6

generally found associated with the above proteins. It is also present in considerable amounts in vitamin-free casein (Labco). Although it exhibits marked acid lability when in the free state, the activity of casein is not destroyed unless the casein is completely hydrolyzed, suggesting that the factor is an integral part of the protein and does not become subject to attack until the protein is completely broken down.

*Effect of Reducing and Oxidizing Agents*—The factor is rather labile to reducing and oxidizing reagents. The data are summarized in Table II. In all experiments the material contained 1 unit in 0.45  $\gamma$  and was in a 1 per cent solution.

*Evidence of Acidic Nature of Factor*—With a ten cell electrodialysis apparatus similar in design to the one described by Williams and Waterman (5) and when a 110 volt 50 milliampere direct cur-

rent was applied, the active material was concentrated in the acidic cells. This demonstrates that the acidic properties of the factor are predominant. This conclusion is based on the fact that the current applied was too small to bring about electrolysis. An electrostatic field was produced in which the compounds oriented themselves according to their potentials, which are measures of their acidity or basicity.

The activity was destroyed by esterification with EtOH-HCl at room temperature and regenerated by hydrolysis with sodium carbonate. Only 50 per cent of the activity was recovered. The loss was not due to incomplete hydrolysis of the ester but is attrib-

TABLE II  
*Stability of Factor to Reduction and Oxidation*

Reagent	Treatment	Loss <i>per cent</i>
Pt-H <sub>2</sub>	Atmospheric pressure for 8 hrs. at pH 7.0	48.0
Sodium hydrosulfite (0.04 M)	90° for 1 hr. at pH 3.0 or 7.0	21.0
Ferrous sulfate (0.02 M)	90° " $\frac{1}{2}$ " " " 7.0	23.0
Hydrogen sulfide	1 hr. at pH 3.0	33.0
	1 " " " 7.0	16.0
" peroxide, 1%	90° for 1 hr. at pH 7.0	84.0
" " 0.1%	90° " 1 " " " 7.0	33.0
" " 0.01%	90° " 1 " " " 7.0	None
Potassium ferrieyanide (0.02 M)	90° " 1 " " " 7.0	"

uted to the destruction of the factor by the HCl before esterification. These two experiments lead to the conclusion that the factor is predominantly an acid and that at least part of the acidity is due to a carboxyl group. The inactivity of the ester of this acid is in agreement with other observations that esters are generally unavailable to this organism; *e.g.*, inactivity of esters of pantothenic acid (6) and biotin.<sup>2</sup>

*Evidence for Presence of an Amino Group*—The activity is destroyed by nitrous acid (83 per cent in 1 hour), indicating the presence of a free amino group. The activity is also destroyed by acetic anhydride or benzoyl chloride in the presence of pyridine.

<sup>2</sup> Shull, G. M., Hutchings, B. L., and Peterson, W. H., unpublished data.



However, it has been impossible to regenerate by various techniques of hydrolysis more than a few per cent of the original activity. This resistance to hydrolysis is typical of benzoyl or acetyl groups attached to nitrogen. These reactions indicate, but are not conclusive proof, that there is an amino group in the compound and that it is necessary for biological activity.

*Evidence against Nucleotide Theory*—As has already been mentioned, Stokstad believes the active compound is a nucleotide. If it can be shown that the organic phosphorus present is not necessary for the activity of the compound, it would be evidence against the view that the active compound is a nucleotide. De-

TABLE III  
*Relation of Phosphorus Content to Activity*

Treatment	Unit activity	Phosphorus	Phosphorus liberated		Loss of activity
	$\gamma$ per unit	$\gamma$ per mg.	$\gamma$ per mg.	per cent	per cent
None.....	0.21	33.18			
1 N $\text{H}_2\text{SO}_4$ at 100°					
4 min.....	0.30	33.18	2.08	6.2	29.8
8 ".....	0.325	33.18	5.02	15.1	35.3
16 ".....	0.36	33.18	9.54	28.8	41.6
32 ".....	0.38	33.18	13.30	40.2	44.6
Clarase equivalent to one-half weight of material; pH 4.5 at 40° for 48 hrs.....	0.21	33.18	32.24	97.4	None

struction of the activity with acid and liberation of phosphorus are not necessarily related. Proof of this can be attained by a different method for the liberation of the phosphorus. For this purpose the commercial enzyme preparation "clarase,"<sup>3</sup> which has a high phosphatase content, was chosen. The results of the acid and enzyme hydrolysis studies are summarized in Table III. It can be seen that the acid destruction and phosphorus liberation are not parallel and that the phosphorus does not seem to be necessary for the activity of the compound.

*Biological Importance*—Certain representative lactic acid bacteria were tested to see whether this compound was of general

<sup>3</sup> Takamine Corporation, 132 Front Street, New York.

nutritional importance within this group, the criteria being either that the bacteria needed the compound preformed in the medium or that they had the ability to synthesize it. This was done by measuring the growth obtained in the presence and absence of the factor. Those organisms that grew in the absence of the factor were then autolyzed and assayed. It was found that growth of *Lactobacillus helveticus*, *Lactobacillus delbrückii*, *Propionibacterium pentosaceum*, and *Streptococcus lactis* was stimulated by addition of the factor. Growth of *Bacillus lactis acidi*, *Lactobacillus arabinosus*, *Lactobacillus pentosus*, *Bacillus brassicae*, *Leuconostoc mesenteroides*, and *Lactobacillus gayonii* was not stimulated, but in every case these organisms had synthesized significant amounts of the factor. This suggests that this compound plays an indispensable rôle in the nutrition of these organisms. Recently this fraction has been found to stimulate the growth of the tetanus organism.<sup>4</sup> Mitchell, Snell, and Williams (3) report a slight growth increase when rats were fed concentrates of their active compound. Under very carefully controlled conditions we have not been able to secure any significant differences.<sup>5</sup> The inability to secure a significant response may well be due to bacterial synthesis, as suggested by the Texas group. These facts together with the previous report of Hutchings *et al.* on its rôle in the nutrition of the chick indicate that this factor will probably prove to be of nutritional importance in several fields of biology.

#### DISCUSSION

From the data presented certain tentative conclusions can be drawn. The precipitation, electrodialysis, and esterification studies demonstrate that the active principle is an acid. The destruction with nitrous acid and inactivation on acetylation or benzoylation suggest the presence of an amino group.

The factor is very labile to acid and reducing agents, somewhat more stable to alkali and oxidizing agents. We have not been able to account for any of these properties on the basis of functional groups present.

<sup>4</sup> Personal communication from Dr. J. H. Mueller.

<sup>5</sup> Hutchings, B. L., Bohonos, N., Oleson, J. J., Elvehjem, C. A., and Peterson, W. H., unpublished data.

The fact that activity does not depend on the phosphorus content of the preparation leads to the conclusion that a nucleotide is not the active compound.

#### SUMMARY

A method for the preparation of concentrates of the norit eluate factor is presented. The active principle is an acid and probably contains an amino group. The nutritional importance of the compound is indicated by its indispensable rôle in the nutrition of certain bacteria and its probable requirement by the chick.

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# THE OXIDATION OF PHOSPHOLIPIDS IN THE PRESENCE OF ASCORBIC ACID AND CARCINOGENIC CHEMICALS\*

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The oxidation of phospholipids in the presence of glutathione and cysteine has previously been demonstrated (1, 2). More recently Rusch and Kline have shown that ascorbic acid, thiamine, riboflavin, pyridoxine, and methylene blue will also "catalyze" the oxidation of phospholipids (3). They also reported that the carcinogenic chemicals and related compounds will inhibit the oxidation. Of the various systems studied those containing ascorbic acid showed the most rapid rates of oxidation. Accordingly such systems have been studied in greater detail with particular reference to the chemical changes taking place during the reaction and to the mechanism by which the carcinogenic hydrocarbons inhibit it.

## EXPERIMENTAL

Aqueous solutions of ascorbic acid were added to phospholipid preparations suspended in dilute solutions of hydrochloric acid in the presence or absence of a hydrocarbon, and the rate of oxygen consumption measured in an ordinary Warburg manometric apparatus as described previously (3). The amounts of ascorbic acid remaining and the iodine number of the phospholipid were determined at intervals. In addition, the effects of copper and hydroquinone on the system were measured. The ascorbic acid and the carcinogens used were crystalline commercial products, and the phospholipids were prepared from fresh rat livers. These

\* This investigation was aided by the Jonathan Bowman Fund for Cancer Research.

<sup>†</sup> Finney-Howell Research Fellow.

were dried by mincing with acetone in a Waring blender, centrifuging, and repeating the acetone extraction two or three times. The phospholipids were then extracted with 3 volumes of a 1:4 alcohol-ether mixture and the extraction repeated twice. The solution was filtered and the solvent removed under reduced pressure. The residue was then dissolved in a small quantity of ether and precipitated with acetone, and the latter procedure repeated two or three times. The final precipitate was dried in a high vacuum over phosphoric anhydride and sealed in small evacuated tubes.

For the preparation of the aqueous suspensions of phospholipids, known amounts of the lipid were dissolved in peroxide-free ether and added to a 0.00045 M HCl solution (pH 3.5) contained in Erlenmeyer flasks. Suction was then applied and the ether removed at 50° with vigorous shaking. Uniform phospholipid suspensions were thus obtained. The final pH was approximately 4.0. Hydrocarbons or other carcinogens were introduced into the suspension by dissolving known amounts in ether, mixing thoroughly with the ethereal solution of phospholipid, and then adding the mixture to the HCl solution as just described. The inhibitors were usually present in the ratio of 100  $\gamma$  per 10 mg. of lipid—in other words as 1 per cent of the substrate. The lipid concentrations ranged from 5 to 30 mg. per cc. of the final reaction mixture. The final volume of the lipid suspension was adjusted to correct for volume decreases during the removal of the ether.

Definite volumes of the lipid suspensions were accurately measured into Warburg flasks, and a solution of 0.5 mg. of ascorbic acid added to each flask. Control flasks contained phospholipid alone or phospholipid plus 5  $\gamma$  of copper as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . Each reaction mixture was placed in four different flasks. These were equilibrated for 15 minutes at 38°, after which two of the flasks from each series were removed and the contents immediately dried and stored under a high vacuum over phosphoric anhydride. The oxygen consumption of the remaining two samples was measured for various periods, after which the partially oxidized samples were removed and also dried. For the determination of the iodine number, these lipids were dissolved in ether and precipitated with acetone; usually about 60 per cent of the original phospholipid was recovered, although recovery varied from 30 to 85 per cent. The

higher amounts were obtained when the acetone solution was placed in a refrigerator overnight. The iodine numbers were determined on 5 to 20 mg. samples of the dried phospholipid by Yasuda's (4) micro modification of the Rosenmund and Kuhn-henn method (5). In some experiments the iodine numbers were determined on phospholipid not precipitated with acetone but on samples dried *in vacuo* directly after oxidation. Apparently the phospholipids obtained by this procedure were representative of the total phospholipid of the system, since essentially the same iodine numbers were obtained whether the amount of phospholipid recovered represented 30 or 85 per cent of the original amount present. In addition to the iodine number, other measurements made included the determination of the pH of the system, the amount of inorganic phosphate, and the amount of ascorbic acid present. A glass electrode was used for determination of the pH, and inorganic phosphorus was determined by a spectrophotometric modification of the Fiske-Subbarow method. Ascorbic acid was determined at the equilibration point and at half hour periods for 4 hours by titrations with 2,6-dichlorophenol indophenol at pH 3.5.

The change in iodine number was correlated with the oxygen consumption of the system corrected for the amount needed to oxidize the ascorbic acid to the dehydro form. These measurements were then compared with the theoretical amount of oxygen required to form an initial peroxide at the double bond.

### Results

Typical curves of oxygen consumption, as observed with the small amounts of catalyst used in the present study, are shown in Fig. 1. A very slight but continuous oxygen consumption was observed when lipid alone was being oxidized. The oxygen consumption was increased slightly by the presence of copper and markedly by the presence of ascorbic acid. The total oxygen consumption in 8 hours per mg. of lipid was 3.0 c.mm. when no catalyst was used, 9.0 c.mm. in the presence of 5  $\gamma$  of copper, and 55.1 c.mm. in the presence of ascorbic acid. Since copper has been shown to catalyze the oxidation of various lipids (6) as well as that of ascorbic acid (7), it was necessary to determine the effect of copper on the phospholipid-ascorbic acid system. Oxygen consumption was actually decreased by the addition of copper (Fig. 1).

In another experiment, an attempt was made to inactivate traces of copper by the addition of 3 mg. of glycine, but this had no effect on the oxygen uptake. From these findings, it appeared that the catalytic effect observed was not due to the presence of copper.

Several experiments were performed in order to determine whether most of the oxygen uptake of the system employed was due to the oxidation of the phospholipid or to that of ascorbic acid. In the first place, when ascorbic acid was allowed to autoxidize at pH 3.5, the amount of oxygen consumed was only slightly greater than that required to change it to the dehydro

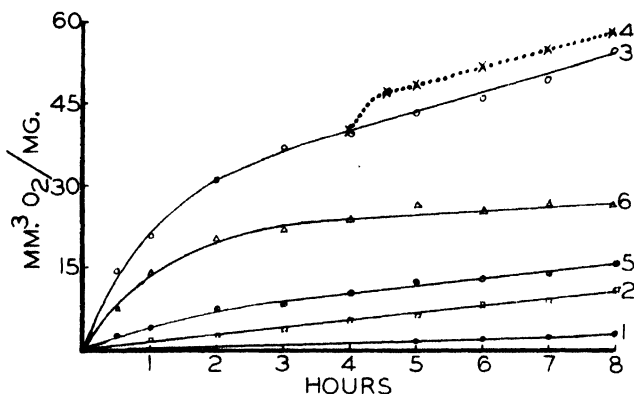


FIG. 1. Phospholipid oxidation in the presence of various substances. Each flask contained 10 mg. of phospholipid in addition to (Curve 1) phospholipid alone, (Curve 2) 5  $\gamma$  of Cu as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , (Curve 3) 0.5 mg. of ascorbic acid, (Curve 4) second addition of 0.5 mg. of ascorbic acid after 4 hours, (Curve 5) 0.5 mg. of ascorbic acid and 5  $\gamma$  of Cu as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , (Curve 6) 0.5 mg. of ascorbic acid and 100  $\gamma$  of benzpyrene.

form (Table I). However, Lyman, Schultze, and King (7) have shown that ascorbic acid will oxidize slowly beyond the dehydro form with the production of  $\text{CO}_2$  if it is in the presence of copper at pH 4.5, but even this would not account for the total oxygen consumption. Furthermore, most of the ascorbic acid disappeared from the system in the first 2 hours (Fig. 2) and the rate of its decrease was inversely proportional to the oxygen uptake. Thus, in a system containing 10 mg. of phospholipid, only one-half of the ascorbic acid (0.25 mg.) was oxidized in 1 hour, while a total of approximately 200 c.mm. of oxygen had been consumed. Only

a small amount of this oxygen could have been utilized in the oxidation of ascorbic acid. In another experiment, an additional 0.5 mg. of ascorbic acid was added to the phospholipid-ascorbic acid mixture after 4 hours, but only a slight increase in oxygen

TABLE I

*Oxygen Uptake with Various Amounts of Phospholipid*

0.00045 N HCl at pH 3.5 to 4. 0.5 mg. of ascorbic acid per flask.

Liver phospholipid	O <sub>2</sub> consumption for 4 hrs.
mg.	c.mm.
0	35
1	78
2	120
3	208
5	260
10	396
26	532

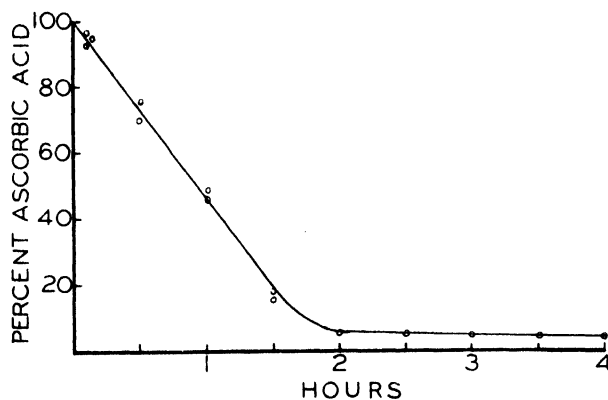


FIG. 2. Titration of ascorbic acid with dichlorophenol indophenol during the oxidation of phospholipid. Each flask contained 10 mg. of phospholipid and 0.5 mg. of ascorbic acid.

uptake was noted (Fig. 1). Since the additional ascorbic acid was present in the side arm before being dumped into the rest of the system, its autoxidation would have added to the total uptake, yet prior to the mixing it was not sufficient to alter greatly the curve as compared to the control. This experiment also suggests



that the reduced form of ascorbic acid was necessary for catalysis. Data are presented in Table I which demonstrate that the amount of oxygen consumed was proportional to the level of phospholipid present. If the ascorbic acid was responsible for all or for most of the oxidation, this would not have been the case. Still more evidence in favor of the oxidation of phospholipid as being responsible for most of the oxygen uptake was demonstrated by the use of benzpyrene. This inhibited oxidation in proportion to the amount of hydrocarbon present (Fig. 3), yet had no effect on the rate of disappearance of the ascorbic acid. Thus it appeared that it was the oxidation of the phospholipid and not that of the catalyst which was interfered with in the presence of benzpyrene.

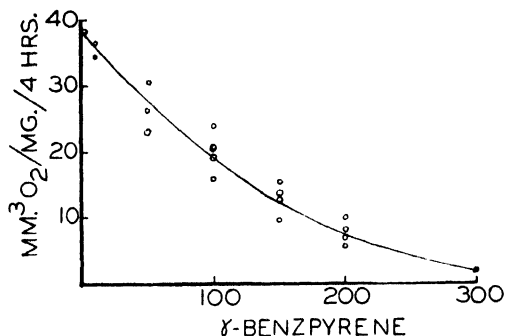


FIG. 3. The effect of varying the amount of benzpyrene on the oxygen consumption of phospholipid. Each flask contained 0.5 mg. of ascorbic acid and 10 mg. of phospholipid with varying amounts of benzpyrene.

In the experiments which continued for 44 hours large amounts of phospholipid (63.85 mg.) were oxidized in the presence or absence of ascorbic acid (Table II). In the presence of ascorbic acid, no latent period was observed and oxygen consumption continued at a fast rate for 24 hours, after which there was a gradual slowing. In the absence of ascorbic acid, an induction period of roughly 8 hours was noted, after which the rate of oxygen uptake became more rapid, and after 18 hours it was approximately the same for both samples. It seemed probable, therefore, that ascorbic acid acted only as a catalyst in accelerating the oxidation of the phospholipids and was not responsible for a marked increase in total oxygen consumption.

Since only a small part of the oxygen consumed was used in the oxidation of ascorbic acid, an attempt was made to account for the remainder of the oxygen required. It was assumed that some of the oxygen was used in oxidizing the unsaturated fatty acids of the phospholipid molecule. To test this correlations were made between the actual oxygen uptake and the calculated amount required to account for the observed change in the iodine number.

TABLE II

*Correlation of Oxygen Uptake with Change in Iodine Number*

0.5 mg. of ascorbic acid per flask. 100  $\gamma$  of inhibitor per 10 mg. of phospholipid.

Phospho- lipid per flask	Inhibitor used	Iodine No.		O <sub>2</sub> uptake per mg. for period oxidized		Oxida- tion	Inhibi- tion
		Before oxida- tion	After oxida- tion	Theo- retical	Actual*		
<i>mg.</i>				<i>c.mm.</i>	<i>c.mm.</i>	<i>hrs.</i>	<i>per cent</i>
13.11		78.4	48.9	26.0	25.2	6	
10.00		91.9	59.7	28.4	25.2	4	
11.67		91.4	56.4	30.9	30.5	4	
31.11		81.4	70.6	9.5	10.2	5	
25.8		96.8	81.3	13.7	13.8	4	
23.3	Benzpyrene	98.6	93.0	4.9	5.4	4	60.7
23.76	Methylcholanthrene	85.0	71.8	11.6	11.1	3	22.3
24.47	Aminoazotoluene	89.1	83.6	4.9	4.0	3	67.9
26.67	Hydroquinone	87.5	85.3	1.9	1.3	3	81.4
32.41	Dibenzanthracene	80.8	61.9	16.7	16.2	6	40.0
63.85†		90.8	65.0	22.8	22.0	44	
63.85		88.9	56.5	28.6	28.4	44	

\* Corrected for oxygen needed to oxidize ascorbic acid to the dehydro form.

† No ascorbic acid added.

That these two values were reasonably close can be seen in Table II. Thus when 13.11 mg. of phospholipid were oxidized for 6 hours in the presence of 0.5 mg. of ascorbic acid, the actual uptake was 25.2 c.mm. This compares favorably to the calculated theoretical value of 26.0 c.mm. To obtain the theoretical figure, corrections were made for the oxygen needed to oxidize the ascorbic acid to the dehydro form. Good correlations were found

between the theoretical and actual uptakes in samples showing inhibition induced by carcinogens and hydroquinone additions. The comparisons obtained with larger amounts of lipid (63.85 mg.) either in the presence or absence of ascorbic acid were also satisfactory.

The phospholipid molecule appeared to remain intact during its oxidation, since no inorganic phosphate could be detected nor was there any change in the pH of the unbuffered substrate at any time during or after prolonged oxidation. However, that some change occurred in the oxidized phospholipid was indicated by the change in color from white to brown. Furthermore, the oxidized samples were not as readily precipitated with acetone and this difficulty was increased somewhat parallel to the degree of oxidation. Cloudy emulsions formed when ether solutions of the oxidized fractions were added to acetone and precipitation occurred only after the material had stood for some time in the cold. Even though the precipitation of the phospholipid was incomplete, all samples were apparently uniform and good correlations between actual and theoretical uptakes could be obtained with these samples as well as on those which were not precipitated. The changes in precipitability in acetone following oxidation may well be due to the addition of oxygen to the fatty acid portion of the phospholipid molecule.

The effect of various inhibitors on the phospholipid oxidation is seen in Table II. Hydroquinone was the most effective inhibitor, followed by aminoazotoluene, benzpyrene, 1,2,5,6-dibenzanthracene, and methyleholanthrene. Each experiment was repeated five to eight times and substantiates results previously published (3). The type of curve obtained in the oxidation of phospholipid containing inhibitors was lower but otherwise similar to the curve observed with the uninhibited oxidation (Fig. 1). To test the effect of different amounts of benzpyrene on the oxygen consumption, the amount of phospholipid was kept constant, but the level of the carcinogen was varied from 10 to 300  $\gamma$  per 10 mg. of lipid. Although greater inhibition was obtained with the higher levels, there was no apparent molar relationship between the amounts used and the percentage inhibition (Fig. 3).

Benzpyrene appeared to interfere in some manner with the

physicochemical properties of the lipid, since following oxidation the character of the acetone precipitate was changed. Thus, the samples containing benzpyrene were not only much darker in color, but in most cases even less recovery could be obtained by acetone precipitation as compared to oxidized samples free of benzpyrene. It appeared that the benzpyrene may have formed some sort of complex with the lipid which tied up the double bonds. In order to test this a system of lipid, ascorbic acid, and benzpyrene was shaken in an atmosphere of nitrogen for 20 hours. Since no change in iodine numbers resulted, it appeared that the effect observed depended on the presence of oxygen.

#### DISCUSSION

The catalytic action of ascorbic acid in the oxidation of phospholipid is unique, since various experiments have demonstrated its antioxygenic capacity. Golumbic and Mattill (8) have reported that ascorbic acid is an effective antioxidant for certain vegetable oils and also observed that it enhances the antioxygenic activity of tocopherols, hydroxychromans, hydroquinones, and related compounds. We have shown that the induction period in the autoxidation of ethyl linoleate has been increased by its presence (9). Others (10-12) have demonstrated that ascorbic acid retards the development of oxidative changes in milk and various other foodstuffs. In considering the different results observed by these workers, as compared to our findings with isolated phospholipids, it should be recalled that our experiments were performed in an aqueous acidified medium. Thus the ascorbic acid was in intimate contact with the phospholipid and also tended to remain in a reduced condition. However, the occurrence of the fatty acids as part of the phospholipid molecule may in itself be sufficient to permit a different reaction.

The mechanism by which hydrocarbons inhibit the oxidation of phospholipids is still unknown. It appears that the mode of action involves the lipid phase of the system rather than the ascorbic acid, since the disappearance of the latter is unaffected by the presence of benzpyrene. Furthermore, our data indicate that the hydrocarbons inhibit the addition of oxygen to the phospholipid (Table II). The importance of this inhibitory action in the mechanism of cancer formation is unknown.

## SUMMARY

1. The oxidation of phospholipids in the presence of ascorbic acid was studied with the aid of the manometric technique, and the effect of carcinogenic chemicals, hydroquinone, and copper on this system was observed.

2. Phospholipid oxidation was catalyzed by the presence of ascorbic acid at pH 4.

3. The catalyzed phospholipid oxidation was inhibited by the presence of carcinogenic chemicals and hydroquinone.

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# THE EFFECT OF SODIUM FLUORIDE AND SODIUM IODOACETATE ON GLYCOLYSIS IN HUMAN BLOOD\*

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It has been generally accepted that a concentration of 1 per cent sodium fluoride is adequate to prevent glycolysis in non-precipitated blood and that no appreciable glycolysis results if the blood proteins are precipitated within 5 minutes after the blood is drawn (1). Determinations of lactic acid in the blood precipitated within 5 minutes or preserved in 1 per cent sodium fluoride have yielded a range of 8 to 20 mg. per cent for normal subjects under basal conditions.

We have had occasion to analyze pyruvic acid and lactic acid on the same blood samples. The analysis of pyruvic acid requires the use of iodoacetate as a stabilizing agent (2). The lactic acid values we observed in blood from normal fasting subjects at rest were found to be appreciably lower than those hitherto reported. We therefore reinvestigated the inhibitory effect of sodium fluoride and iodoacetate on glycolysis in human blood.

## *Methods*

*Drawing of Blood*—The blood was drawn from the antecubital vein without stasis. If stasis were required to puncture the vein, the blood was drawn after the pressure on the arm had been released. The sodium iodoacetate used was prepared from iodoacetic acid (Eastman Kodak) (3) as a 30 per cent solution. The iodoacetate solution was measured into the syringe used to draw the blood and mixed with the blood in the syringe before

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delivery into the sampling bottles. The glucose and lactic acid values observed were corrected for the dilution. The sodium fluoride was added to the sampling bottles as a 4 per cent solution and then dried to a powder over a hot-plate. 0.2 per cent potassium oxalate was used for all blood samples in addition to the preservative used.

*Analytical Methods*—The blood sugar was estimated in an aliquot of 0.2 cc. of blood (2 cc. of blood filtrate) by a modification (4) of the Hagedorn-Jensen method, 0.002 instead of 0.005 N sodium thiosulfate being used for the final titration. The samples were analyzed in duplicate and the duplicate determinations never varied more than 0.7 mg. per cent. Non-fermentable reducing substances were determined by yeast fermentation (5).

For the lactic acid determination 15 cc. of blood were used. A 20 cc. aliquot of the carbohydrate-free filtrate (representing 1.6 cc. of blood) was determined according to Wendel's modification (6) of the method of Friedemann, Cottonio, and Shaffer (7). All determinations were made in duplicate or triplicate.

In eleven analyses known amounts of lithium lactate were added, corresponding to a lactic acid concentration varying from 3.0 to 12.5 mg. per cent. In six instances the blood contained 0.2 per cent oxalate and recoveries varied from 92 to 104 per cent (average, 97 per cent). In the other five analyses the blood contained 0.2 per cent potassium oxalate, 1 per cent sodium fluoride, and 1 per cent sodium monoiodoacetate and 93 to 98 per cent (average, 95 per cent) of the added lactic acid was recovered.

*Experimental Method*—In the present experiments we have estimated the glycolysis in human blood over a period of 4 hours. The blood was drawn without stasis and added to various concentrations of iodoacetate or sodium fluoride. The blood proteins of an aliquot were precipitated (8) immediately and the filtrate was analyzed for glucose and lactic acid. A second aliquot was precipitated 4 hours later.

Observations were made on the changes in non-glucose reducing substances with and without iodoacetate and sodium fluoride. We have repeatedly observed that there was no change in the non-glucose reducing substances of the blood during the 4 hour period of the experiment. We therefore feel that the values observed in the following experiments represent changes in the true glucose of the blood.

## Results

*Effect of Sodium Fluoride on Glycolysis*—The changes in the non-precipitated blood during 4 hours after withdrawal from the vein are presented in Fig. 1, A to E. It may be seen that various concentrations of sodium fluoride up to 2 per cent did not prevent completely the decrease in glucose nor the rise in lactic acid of

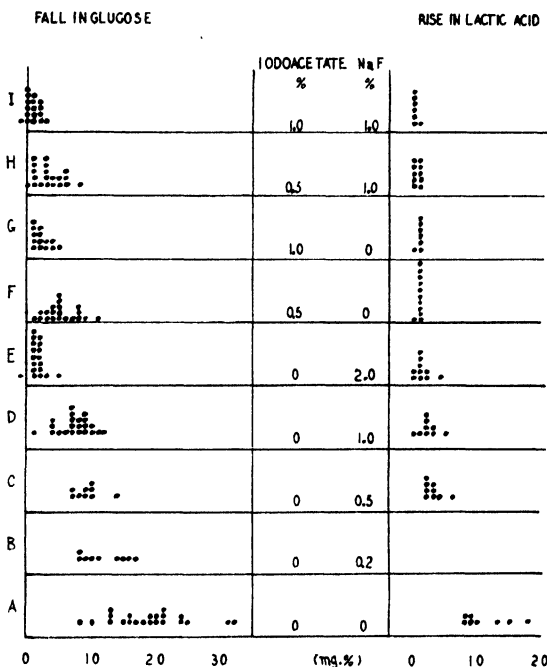


FIG. 1. Effect of various concentrations of sodium fluoride or sodium iodoacetate, and concentrations of both on the changes in sugar and lactic acid of human blood on standing at 20° for 4 hours. (All samples contain 0.2 per cent oxalate.)

the non-precipitated blood samples. In practically all cases the loss of glucose exceeded the rise of lactic acid. The averages of each group were analyzed by Fisher's method (9) and were found to be statistically significant. In order to test the effect of sterility on glycolysis, we have analyzed a series of blood samples preserved with 1 per cent sodium fluoride in which one aliquot was kept in sterile containers, and a second aliquot was left open to



the air. No difference was found between the fall in glucose concentration in the two sets of specimens in 4 hours.

These observations do not agree with the results hitherto reported on the effect of sodium fluoride on glycolysis. Most of the previous investigators have estimated the rate of glycolysis from analyses of blood sugar values (10–15). These authors have used methods for blood sugar estimation inadequate to detect changes of the order of magnitude reported in this paper.

*Effect of Monoiodoacetate on Glycolysis*—It may be seen (Fig. 1, *F* and *G*) that the disappearance of glucose was inhibited by both 0.5 and 1.0 per cent iodoacetate, but neither concentration

TABLE I

*Effect of 1 Per Cent Iodoacetate Plus Sodium Fluoride on Glycolysis within 5 Minutes after Blood Was Drawn*

Sample No. ....	1	2	3	4	5	6	7	8	9
1% iodoacetate + 1% NaF .....	5.6	5.4	7.0	5.9	6.9	8.2	6.4	7.4	6.5
None .....	6.6	6.6	8.1	7.4	8.7	10.0	7.2	7.4	7.0
Sample No. ....	10	11	12	13	14	15	16	17	18
1% iodoacetate + 1% NaF .....	7.6	4.9	8.4	8.4	18.3*	7.9	5.3	6.8	5.8
None .....	8.5	6.2	10.4	8.0	19.5*	9.3	6.5	7.2	7.2

\* No basal conditions.

of iodoacetate completely stopped the glycolysis. In both there was a significant loss of glucose and rise in lactic acid in 4 hours.

*Effect of Iodoacetate Plus Fluoride on Glycolysis*—The rate of glycolysis was studied on a series of blood samples preserved with 1 per cent sodium fluoride plus either 0.5 or 1 per cent iodoacetate (Fig. 1, *H* and *I*). There was a perceptible diminution of the rate of glucose disappearance in 1 per cent sodium fluoride plus 0.5 per cent iodoacetate and practically complete preservation of the blood sugar and lactic acid could be accomplished with 1 per cent sodium fluoride plus 1 per cent iodoacetate. The inhibition of lactic acid production was observed with the 0.5 per cent iodoacetate plus 1 per cent sodium fluoride.

*Lactic Acid Values in Blood Precipitated within 5 Minutes*—Blood was drawn through a single needle into a dry syringe and added to enough oxalate in a sampling bottle to make the concentration 0.2 per cent. An additional sample was drawn through the same needle containing a sufficient amount of iodoacetate to make a 1 per cent solution, and added to a sampling bottle with sufficient sodium fluoride and oxalate to give a final concentration of 1 per cent sodium fluoride and 0.2 per cent potassium oxalate. Both samples were precipitated within 5 minutes and lactic acid analyzed. The data are presented in Table I. It may be seen that the lactic acid values of most of the bloods containing iodoacetate and sodium fluoride were appreciably lower than the sample without the preservatives, indicating that some glycolysis did occur in the first 5 minutes.

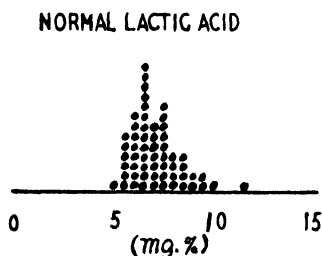


FIG. 2. Lactic acid concentration of normal human blood

*Normal Lactic Acid Values in Blood*—It has been generally accepted that glycolysis can be prevented if sufficient sodium fluoride is added to make a 1 per cent solution. In the present report our observations do not confirm the previous findings, and we believe that the lactic acid values reported previously have at least two sources of error. The conversion of pyruvic acid to lactic acid occurs so rapidly that the ordinary method of drawing blood in a dry syringe would introduce an error regardless of the type of preservatives used (2, 16). In addition we have observed a perceptible rise of lactic acid in blood containing 1 per cent sodium fluoride before protein precipitation. We have therefore analyzed a large series of blood samples from resting patients for lactic acid, using the following precautions. The observations were made on patients at least 14 hours after the last food intake,

and after a rest of at least 1 hour in bed. Blood was drawn without stasis from the antecubital vein into a syringe filled with sufficient iodoacetate to make a 1 per cent solution. The blood was delivered into a sampling bottle with sufficient oxalate and fluoride to give a final concentration of 0.2 per cent oxalate and 1 per cent fluoride. The proteins were precipitated (8) within 5 minutes and the lactic acid analyzed. The results are presented in Fig. 2. It may be seen that the bulk of the determinations falls between 5 and 10 mg. per cent. The average of the 58 determinations was 7.0 mg. per cent.

#### SUMMARY

1. The rate of glycolysis in human blood was estimated from the changes in glucose and lactic acid. Either iodoacetate or sodium fluoride alone did not stop glycolysis completely. Complete inhibition of glycolysis was observed with a mixture of 1 per cent sodium fluoride and 1 per cent iodoacetate.

2. When suitable precautions were used, it was found that the normal lactic acid values in human blood ranged from 5 to 10 mg. per cent.

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## THE DETERMINATION OF CEREBROSIDES

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Cerebrosides are determined through measurement of the galactose set free by acid hydrolysis. In recent years most workers have employed the method of Kimmelstiel (1), or a modification thereof, and have used the procedure of Hagedorn and Jensen (2) for analysis of the galactose.

Miller and Van Slyke (3) called attention to three disadvantages in the method of Hagedorn and Jensen referable to the titration by difference of the ferricyanide present before and after reduction by the sugar. To avoid these drawbacks Miller and Van Slyke proposed a procedure based on direct titration with ceric sulfate of the ferrocyanide formed. Also by replacement of the zinc deproteinization technique with a modification of the cadmium precipitation procedure of Fujita and Iwatake (4) the non-fermentable reducing material in blood filtrates was reduced to practically zero.

In selecting a method for the determination of cerebrosides in brain extracts we decided to incorporate the procedure of Miller and Van Slyke into the Kirk (5) modification of Kimmelstiel's method. The procedure involves the following main steps: (a) hydrolysis, (b) neutralization, (c) precipitation of interfering substances, (d) oxidation with ferricyanide, and (e) titration of the ferrocyanide formed with ceric sulfate. No difficulty was experienced with the determination of galactose by the Miller and Van Slyke procedure at steps (d) and (e), but much trouble was encountered in the preceding steps, especially in the hydrolysis. This experience might have been anticipated from the fact that almost everyone who has determined cerebrosides has employed a different procedure for hydrolysis. The difficulties attending cerebroside analysis are exemplified in a recent paper by Klenk

and Schumann (6) who report values from 10 to 15 per cent too low, obtained with a new and unpublished method in which 10 per cent hydrochloric acid is used for hydrolysis.

In the search for optimal conditions 170 samples of cerebrosides have been analyzed with many different procedures and the recovery of 68 samples added to lipid extracts has been measured. Hydrolysis with hydrochloric acid (5) could not be combined with the cadmium clarification technique (4) at step (c) because, as would be expected, cloudy filtrates were obtained or cloudiness developed at step (d). With 3 N sulfuric acid instead of hydrochloric acid clear filtrates were obtained but the filtration through cotton was slow. Some improvement was achieved by centrifuging and filtering the supernatant suspension but later this cumbersome procedure was replaced by filtration through paper, with which clear filtrates, rapid filtration, and low blanks (if the paper is adequately washed) are obtained.

Unfortunately this procedure could not be used because values consistently 10 to 15 per cent too high were observed when galactose was heated with 3 N sulfuric acid for 10 minutes in a boiling water bath. This result is not necessarily in disagreement with the fact, as pointed out by Thierfelder and Klenk (7), that the recovery of cerebrosides with the method of Loening and Thierfelder (8) (3 hours heating with 3 N sulfuric acid) is about 10 per cent too low. With 1 N sulfuric acid the recovery of galactose was quantitative even after 24 hours heating, but the theoretical values could not be obtained on cerebrosides. With 10 minutes heating with 1 N sulfuric acid only about 67 per cent of the calculated amount of cerebroside was recovered; after 24 hours the yield was about 90 per cent.

We were forced by this result to return to the use of hydrochloric acid for hydrolysis, and, therefore, to give up the cadmium (4) in favor of the zinc (2, 5) clarification technique. However, the filtration through washed paper was retained, as it was found to be faster than filtration through cotton and to give as uniform results.

It has been impossible to confirm the finding of Kirk (5) that quantitative hydrolysis of cerebrosides may be achieved by heating for 10 minutes with 3 N hydrochloric acid in a boiling water bath in a tube covered by a funnel with a sealed tip. In thirteen

determinations of pure cerebroside with this procedure the maximal recovery was 88.9 per cent (average 82.8 per cent). When the time of heating was extended to 0.5, 1, or 2 hours with the other conditions the same, values within  $\pm 2$  per cent of the theoretical were observed in ten of twenty samples, but in the others the recoveries were from 4 to 17 per cent too low. In several instances one of two duplicate determinations gave the correct analysis, while the other, carried through at the same time under exactly the same conditions, yielded a low result. No reason for this variable behavior has been found. Galactose was recovered quantitatively, and about the same proportion of low values for cerebroside was obtained with each of the three periods of heating. Both correct and low values were yielded by each of the three cerebroside preparations (*cf.* foot-notes to Table I) used.

Incomplete emulsification was a possible explanation of the variation. It is difficult to evaluate this factor in the hydrolyzed samples because of the liberation of fatty acids, but in unhydrolyzed samples, heated with water (1, 5), poor emulsification was frequently observed. Hence the effect of "purified sodium alkyl sulfate"<sup>1</sup> as an emulsifying agent was tried. Although it had no demonstrable effect on the recovery of cerebroside, it undoubtedly improved the emulsification and, therefore, has been retained in the final procedure, with the thought that it could do no harm (it gives no blank) and might be of benefit in the analysis of unknown lipid extracts.

In the original procedure of Kimmelstiel (1) hydrolysis of cerebroside was accomplished by heating with 3 *N* hydrochloric acid in a tightly stoppered 50 cc. flask for 15 to 18 minutes in an oven at 112°. With this procedure (15 minutes in an oven at 110–115° in a stoppered 10 cc. volumetric flask) very low values (about one-third of the theoretical) were obtained. When the flasks were placed in a previously heated sand bath in the oven for 15 minutes, the recovery was about 90 per cent. This result was ascribed to incomplete hydrolysis, since recovery of galactose was quantitative under the same conditions;<sup>2</sup> hence the time of

<sup>1</sup> The "purified sodium alkyl sulfate" was supplied by The Procter and Gamble Company, Chemical Division, Ivorydale, Ohio.

<sup>2</sup> At a later date the recovery of galactose was determined again after heating with 3 *N* hydrochloric acid in a stoppered 10 cc. flask for 15 minutes

TABLE I

*Determination of Cerebrosides*

Unhydrolyzed samples (see the text) were not included in these determinations, as it had been shown that there was no reduction of ferri-cyanide by these cerebrosides.

Substance taken		Galactose	Cerebroside	
			Found	Recovery
	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
Brain cerebroside*	1.388	20.8	1.312	94.5
	1.388	21.5	1.356	97.7
	1.388	21.9	1.382	99.6
	1.388	21.9	1.382	99.6
Phrenosin†	1.282	21.1	1.247	97.3
	1.282	22.1	1.306	101.9
	1.282	22.1	1.306	101.9
	1.282	22.2	1.312	102.3
	1.282	21.5	1.270	99.1
	1.282	21.3	1.258	98.1
	1.282	22.1	1.306	101.9
	1.282	22.0	1.300	101.4
	1.282	22.1	1.306	101.9
	1.282	22.1	1.306	101.9
Kerasin†	1.422	21.8	1.396	98.2
	1.422	21.6	1.384	97.3
	1.360	21.9	1.342	98.7
	1.360	22.1	1.354	99.6
	1.360	22.9	1.403	103.2
	1.360	22.6	1.385	101.8
Average.....			99.8 ± 2.23	

\* The brain cerebroside was prepared by the method of Page (10). It contained 0.13 per cent phosphorus for which correction was made as sphingomyelin. A theoretical galactose content of 22 per cent was assumed in calculation of the recovery.

† The phrenosin and kerasin were prepared by Dr. Erwin Chargaff. They were free of phosphorus. The theoretical galactose concentrations are 21.7 and 22.2 per cent respectively.

in a sand bath in an oven at 111–113°. The average recovery was 96.1 per cent, with a minimum of 93.4 per cent. From this result it is possible that the low cerebroside values represented, at least in part, a destruction of galactose. Our use of a smaller flask may have been responsible for our failure to confirm Kimmelstiel's findings, although it is difficult to explain on this basis the very low recovery when the flasks were placed in the oven without immersion in hot sand.

heating was increased to 30 minutes with the other conditions the same. In twelve of twenty-three samples of cerebrosides the values were within 3 per cent of the theoretical; in the others the recoveries were from 4 to 14 per cent too low. Variation in the oven temperature was not responsible, since in several instances

TABLE II

*Recovery of Cerebrosides Added to Brain Lipid Extracts*

1 cc. of extract was taken for each determination.

Sample added		Cerebroside found		Recovery
		In extract + added sample	In extract alone*	
	mg.	mg.	mg.	per cent
Brain	1.388	2.048	0.678	98.7
	cerebroside 1.388	2.066	0.678	100.0
Phrenosin	1.282	1.899	0.655	97.0
	1.282	1.899	0.655	97.0
	1.282	1.950	0.742	94.2
	1.282	1.932	0.655	99.6
	1.282	1.932	0.655	99.6
	1.282	1.932	0.655	99.6
	1.282	1.858	0.632	95.6
	1.282	1.899	0.632	98.8
	1.282	1.872	0.632	96.7
	Kerasin 1.422	2.097	0.657	101.3
Kerasin	1.422	2.061	0.657	98.7
	1.360	2.003	0.662	98.6
	1.360	2.003	0.662	98.6
	1.360	1.986	0.698	94.7
	1.360	2.032	0.698	98.1
Average.....				98.0 $\pm$ 1.87

\* Unhydrolyzed samples were not included, since the value would cancel out in calculation of the recovery.

good and bad recoveries were obtained in duplicate samples carried through side by side under exactly the same conditions. Destruction of galactose occurring in some samples but not in others was probably responsible for the low values, since in four samples of galactose heated for 30 minutes under the same conditions yields of 99, 95, 91, and 98 per cent respectively were observed.



A recovery of about 80 per cent was yielded by both cerebrosides and galactose when heated in the oven for 1 hour.

Presumably because of an experience similar to ours, Erickson *et al.* (9) intensified Kirk's conditions for hydrolysis; the concentration of hydrochloric acid was increased from 3 N to 6 N, the time of heating in boiling water was increased from 10 to 15 minutes, and tightly stoppered cylinders instead of open (funnel at top) tubes were used. With this procedure 97 and 98 per cent recovery was obtained in two samples of cerebrosides, but the recovery of cerebrosides added to brain extracts was 10 and 15 per cent too low in two determinations. Furthermore values 10 to 15 per cent too low were usually observed when galactose was carried through this procedure.

From the foregoing experience it appears that under mild conditions, as in the Kirk procedure, there is danger of incomplete hydrolysis, while under more severe conditions, as in the Kimmelsiel and Erickson methods, there is danger of destruction of galactose. In an attempt to find suitable intermediary conditions hydrolysis was carried out with 3 N hydrochloric acid in stoppered 10 cc. volumetric flasks for 30 minutes in boiling water. The results were mostly acceptable, though a few low values (up to 10 per cent) were obtained. An increase in the time of heating to 45 minutes yielded satisfactory results (Tables I and II).

#### EXPERIMENTAL

##### *Reagents—*

6 N hydrochloric acid.

Approximately 6 N sodium hydroxide (carbon dioxide-free). The titer should be slightly higher than 6 N.

Approximately 1 N hydrochloric acid.

0.1 N sodium hydroxide.

0.1 per cent chlorophenol red in water. A few drops of 0.1 N sodium hydroxide are necessary to dissolve the indicator.

0.1 per cent alkyl sulfate<sup>1</sup> solution.

4.5 per cent zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ).

Alkaline ferricyanide solution (3).

Approximately 18 N sulfuric acid (3).

Stock solution of ceric sulfate, prepared according to the method of Miller and Van Slyke (3) except that it is made 0.15 N.

0.003 N ceric sulfate solution. 2 cc. of stock solution and 5 cc. of 18 N sulfuric acid are diluted to 100 cc.

0.1 per cent solution of alkali-fast erio green.

Washed filter paper, 7 cm. Either Whatman No. 41 or Seavy's No. 106<sup>3</sup> paper is extracted in a continuous extractor (11) in a Pyrex percolator with water until the blank becomes constant (usually 35 to 40 hours).

### *Method*

(a) *Hydrolysis*—Equal portions of a lipid extract containing not over 2.8 mg.<sup>4</sup> of cerebrosides are pipetted into two 10 cc. volumetric flasks, with care that the entire sample is at the bottom of the flask. After removal of the solvent 2 cc. of alkyl sulfate solution are added to each flask, and also to two other flasks to be carried through the procedure as blanks. The flasks are stoppered and placed in boiling water for 5 to 10 minutes, with occasional swirling to emulsify the lipids. To one of the flasks containing lipids and to one of the blank flasks are added 2 cc. of 6 N hydrochloric acid; 2 cc. of water are added to each of the others. The flasks are swirled to mix the contents, stoppered tightly, and heated in boiling water for 45 minutes.

(b) *Neutralization*—After cooling, 1 drop of the chlorophenol red<sup>5</sup> solution and 2 cc. of 6 N sodium hydroxide are added to the flasks containing hydrochloric acid. 1 N hydrochloric acid is added dropwise until the solution is acid (yellow) and the color is finally brought just to a definite purple by careful addition of 0.1 N sodium hydroxide. 2 cc. of water are added to each of the other flasks to make the volumes approximately the same in all.

(c) *Clarification*—After cooling, 0.5 cc. of the zinc sulfate solution is added to each flask followed by 2 cc. of 0.1 N sodium hydroxide. The samples are shaken vigorously. From this point either of two procedures may be used. (a) Water is added to the mark and the suspension is mixed and filtered through washed

<sup>3</sup> This inexpensive, American made paper may be obtained from M. J. Seavy, 30 Church Street, New York.

<sup>4</sup> Larger amounts may be analyzed by increasing the quantity of ferricyanide at step (d).

<sup>5</sup> Phenol red (5) cannot be used, because it obscures the end-point in the titration with ceric sulfate.

paper into a small flask or test-tube. An aliquot (7 or 8 cc.) is pipetted into a special test-tube (3) or 50 cc. Erlenmeyer flask and water is added to make the total volume about 18 cc. (b) The solution is transferred quantitatively through a washed filter into a test-tube (3) or 50 cc. Erlenmeyer flask (four washings with 3 cc. portions of water—total volume about 18 cc.). With this procedure it is not necessary to use volumetric flasks for hydrolysis.

The filtrates from unhydrolyzed lipid samples, *i.e.* those heated without hydrochloric acid, are usually opalescent or slightly cloudy. They may be clarified somewhat by refiltration through the same paper, but this is unnecessary, unless the cloudiness is marked, because it does not affect the result except in so far as it tends to obscure the end-point in titration with ceric sulfate.

(d) *Oxidation with Ferricyanide*—2 cc. of the ferricyanide solution are added to each of the tubes or flasks which are fastened in a rack and heated in boiling water according to the directions of Miller and Van Slyke ((3) p. 590).

(e) *Titration*—The titration with ceric sulfate is carried out as described (3) except that alkali-fast erio green is used as an indicator instead of setopaline C, which is not available at present.<sup>6</sup>

### Calculation

The factor for galactose was constant over the range from 0.1 to 0.6 mg. The average of thirty-three determinations was  $0.147 \pm 0.002$  (s.d.) mg. of galactose per 1 cc. of 0.003 N ceric sulfate. The quantity of cerebroside is calculated from the following equation,  $4.55^7 \times 0.147((A - B) - (C - D)) = \text{mg. of cerebroside}$ , in which A, B, C, and D are the volumes of 0.003 N ceric sulfate solution required to titrate the hydrolyzed lipid sample, hydrolyzed blank, unhydrolyzed lipid sample, and unhydrolyzed blank respectively.

<sup>6</sup> Although alkali-fast green is less sensitive than setopaline C (3), we obtained equally good results with the two indicators.

<sup>7</sup> This factor is used on the assumption that approximately equal quantities of phrenosin and kerasin are present. The factor for phrenosin is 4.6 and for kerasin it is 4.5.

## SUMMARY

The method of Miller and Van Slyke (3) for determination of sugar by direct titration with ceric sulfate was applied to the analysis of cerebrosides.

Difficulties were encountered in the hydrolysis of cerebrosides when carried out according to published procedures for their quantitative determination. Under mild conditions (5) hydrolysis is incomplete, while under more severe conditions (1, 9) destruction of galactose may occur.

A procedure for the quantitative determination of cerebrosides is described.

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# THE PERMEABILITY OF HUMAN ERYTHROCYTES TO RADIOACTIVE CHLORIDE, AND TO BROMIDE AND IODIDE\*

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In a previous study with radioactive isotopes the permeability of human erythrocytes to potassium, sodium, and inorganic phosphate was determined (1). The present report deals with the permeability to chloride, studied with a radioactive isotope,  $\text{Cl}^{38}$ . The permeability of erythrocytes to bromide and to iodide was also determined, and the results compared.

## *Methods*

Blood from subjects without demonstrable disease affecting the red blood cells was used. Lithium chloride or potassium chloride rendered radioactive by deuteron bombardment within the cyclotron was employed. Non-radioactive sodium bromide and sodium iodide were used in experiments dealing with these anions. Blood aerobically defibrinated was divided into two portions, one serving as a control. In the other portion the cells were separated by centrifugation, and the dried salt was dissolved in the supernatant serum, after which the cells were remixed with the serum. Both samples were then introduced into 700 cc. glass tonometers con-

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taining air mixed with  $\text{CO}_2$  at a partial pressure of 40 mm. of mercury. The tonometers were placed in a constant temperature water bath and rotated continuously. The blood spread out in a thin layer over the inner surface of the glass, affording a maximal opportunity for interchange between the cells and the serum. At the end of the period, usually 10 minutes, the samples were removed from the tonometers and the cell volume of each was determined with Daland tubes. Part of each specimen was reserved for whole blood analyses, while the remainder was centrifuged and the serum drawn off. Chloride was determined by the method of Van Slyke (2), bromide and iodide by a modification of that of Brodie and Friedman (3).

Radioactivity was determined by means of a Geiger-Müller counter in a manner previously described (1). In view of the rapid radioactive decay of  $\text{Cl}^{38}$  (half life 37 minutes), all counts had to be multiplied by an appropriate factor to adjust them to a common point of time. These factors were read off from the curve of radioactive decay of  $\text{Cl}^{38}$ . Lithium, with a half life of 0.88 second, had deteriorated within 3 hours to such an extent that its radioactive contribution was negligible. Whenever potassium chloride was used, the counting was repeated on the following morning, by which time  $\text{Cl}^{38}$  had completely deteriorated and all measurable radioactivity was due to  $\text{K}^{42}$  alone (half life 12.4 hours). These counts, adjusted for decay of  $\text{K}^{42}$  since the previous day, represented the portion of the original radioactivity due to  $\text{K}^{42}$ . By subtracting these values from the total original counts the radioactivity due to  $\text{Cl}^{38}$  alone was determined.

Units were chosen in each experiment so that the specific radioactivity (radioactivity divided by concentration of chloride) of the chloride in serum water was unity. Were complete simple equilibrium to be established between the chloride in cells and in serum, the specific radioactivity in cell water would therefore also be unity. Conversely, values of cell specific radioactivity less than unity would indicate failure to achieve such equilibria.

Cell concentrations were obtained by the formula,

Concentration in cells =

$$\frac{(\text{whole blood concentration}) \text{ minus } ((\text{serum concentration}) \times (1 \text{ minus cell volume}))}{\text{cell volume}}$$

Serum and whole blood water contents were determined by weighing before and after drying. The cell water content was then calculated by the formula given above. All concentrations are expressed in terms of serum water or of cell water.

### Results

In Table I are presented the results of three experiments with potassium chloride and seven with lithium chloride. In three

TABLE I  
*Distribution of Chloride between Cells and Serum*

Ex- per- iment No.	Salt used	Tem- pera- ture	Amount		Time	Cell Vol- ume 1	Cell Vol- ume 2	Chloride				Ratio, cell to serum chloride distribu- tion coeffi- cients (d)/(c) (b)/(a)	Cell specific radio- activity* Cl <sup>36</sup> cells Cl cells
								Serum water 1 (a)	Cell Water 1 (b)	Serum Water 2 (c)	Cell Water 2 (d)		
		°C.	mM	min.	per cent	per cent	mM per l.	mM per l.	mM per l.	mM per l.			
1a	LiCl	38	55.0	15	35.6	29.8	108.6	67.0	178.3	102.5	0.93	0.99	
1b	"	38	55.0	60	36.9	30.3	108.4	69.7	176.1	112.7	1.00	1.09	
2	"	38	36.6	10	41.1	35.4	106.3	70.3	148.0	97.3	0.99	0.80	
3	"	38	30.0	10	41.8	37.6	111.1	76.3	148.3	103.8	1.02	0.96	
4	KCl	38	18.8	10	52.6	49.1	111.4	69.7	141.3	79.5	0.90	1.19	
5	"	38	38.7	10	44.1	39.0	105.0	74.4	149.7	103.7	1.02	0.94	
6	LiCl	7	21.0	10	40.4	36.9	102.8	81.4	127.0	101.0	1.01	1.16	
7	KCl	7	26.2	10	39.2	35.0	99.6	81.9	137.0	88.7	0.79	0.90	
8	LiCl	38	39.4	10	43.4	36.8	109.5	79.5	159.5	105.8	0.91	1.00	
9	"	38	29.2	10	40.8	36.1	107.7	73.5	146.0	90.3	0.91	1.11	
10	"	7	27.0	10	46.8	43.3	109.2	82.3	142.1	110.3	1.03	1.00	

\* The specific radioactivity in serum is taken as unity.

experiments the temperature was 7°, in the remainder 38°. In Experiments 1a and 1b the samples were equilibrated 15 minutes and 60 minutes respectively, while the remainder of the experiments lasted only 10 minutes. There is no evident correlation of the results which were obtained with the salt used, with temperature, or with the time of equilibration. In all instances the specific radioactivity of the chloride in the cells (last column) fell between 0.80 and 1.20. Values within this range are not significantly different from unity, since the radioactivity of whole blood



and of serum is only measured with an accuracy of 5 per cent, and that of the cells is calculated in such a way as to exaggerate any error 3- or 4-fold. The distribution ratio of chloride between cell water and serum water was usually not much changed by the addition of salt. The ratios of the final to the initial cell to serum distribution coefficients (next to the last column) fell between 0.90 and 1.03 in all but one instance, Experiment 7. Assuming an accuracy of 1 per cent in the chloride method and in the hematocrit determination, any ratio lying between 0.90 and 1.10 is not certainly different from unity. This means that added chloride behaves in general as if it were distributed between cells and serum

TABLE II

*Distribution of Bromide between Cells and Serum*

NaBr only was used. All experiments were carried out at 38°.

Ex- peri- ment	Corre- spond- ing No. of experiment of Table I	Amount	Time	Initial total halide		Final total halide		Bromide		Ratio, cell to serum total halide distrib- ution coeffi- cients (d)/(c) (b)/(a)	Ratio, bromide in total halide of cells to that in total halide of serum (f)/(d) (e)/(c)
				Serum Water 1 (a)	Cell Water 1 (b)	Serum Water 2 (c)	Cell Water 2 (d)	Serum Water 2 (e)	Cell Water 2 (f)		
		mM	min.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.		
A	None	53.6	180	109.8	76.4	178.7	113.2	60.9	50.9	0.91	1.34
B	"	61.6	180	105.0	63.9	186.6	107.2	80.3	57.3	0.95	1.24
C	2	36.7	10	106.3	70.3	154.7	90.5	44.6	35.2	0.89	1.35
D	3	26.0	10	111.1	76.3	146.4	90.0	29.3	26.3	0.99	1.46

in proportion to the amounts of chloride originally there. This is accomplished in part through a shift of water from cells to serum, as evidenced by the change in cell volume.

In Table II are presented the results of four experiments with sodium bromide. In the last column the proportion of halide present as bromide in the cells is compared with the proportion present as bromide in the serum. The ratio between these two fractions should be unity if bromide distributes itself indifferently through the entire halide of the system, just as in fact the "specific radioactivity" of chloride in the cells (Table I) is unity. The ratio is, however, clearly greater than unity, ranging from 1.24 to

1.46. The ratio is as high after 10 minutes as after 3 hours. Entirely comparable results were obtained with sodium iodide (Table III); here the corresponding ratio ranges from 1.20 to 1.56. In both the bromide and the iodide experiments the final distribution of total halide is such that the distribution ratios between the concentration in cell water and the concentration in serum water are usually but little altered. Thus the ratio of the final to the initial cell to serum halide distribution ratios (Tables II and III, the next to the last columns) ranged between 0.89 and 0.99 in the four bromide experiments and between 0.98 and 1.19 in the four iodide experiments. As in the chloride experiments, values

TABLE III  
*Distribution of Iodide between Cells and Serum*

NaI only was used. All experiments lasted 10 minutes at 38°.

Ex- per- iment	Cor- re- sponding No. of experi- ment of Table I	Amount	Initial total halide		Final total halide		Iodide		Ratio, cell to serum halide dis- tribution coefficients $\frac{(d)/(c)}{(b)/(a)}$	Ratio, iodide in total halide of cells to that in total halide of serum $\frac{(f)/(d)}{(e)/(c)}$
			Serum Water 1 (a)	Cell Water 1 (b)	Serum Water 2 (c)	Cell Water 2 (d)	Serum Water 2 (e)	Cell Water 2 (f)		
		mM	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.		
A	8	27.6	109.5	79.5	141.5	104.0	35.8	32.0	1.01	1.22
B	9	33.2	107.7	73.5	148.5	99.4	35.9	28.8	0.98	1.20
C	10	22.8	109.5	82.3	140.5	106.0	31.6	37.6	1.00	1.56
D	None	29.8	102.8	68.5	133.0	105.5	32.3	33.8	1.19	1.32

lying between 0.90 and 1.10 are not certainly different from unity. Therefore in three out of four experiments in both the bromide and the iodide groups this ratio was essentially unity, while in one experiment in each group it was not. No explanation for these variations in distribution is apparent. This behavior is entirely comparable with that of chloride when LiCl or KCl is added to blood (Table I), and is similarly accompanied by a shift of water from cells to serum.

#### DISCUSSION

These experiments demonstrate that chloride exchange across the red blood cell membrane is so rapid and so complete that at the

end of 10 minutes chloride added to the serum has completely interchanged with all the chloride within the cells. This is a much greater interchange than would be necessary to produce the small net shifts of chloride which occur. Whatever the explanation may be for the characteristic manner in which chloride distributes itself between cells and serum, clearly obstruction by the membrane to the movement of the chloride ion plays no rôle. This behavior is very different from that of potassium, which crosses the membrane so slowly that equilibrium is not attained after several hours (1, 4). Inorganic phosphate may also cross the red cell membrane (1), but it does so only at 38°. At 7° the passage is too slow to detect. Temperature, however, has no apparent effect on chloride transfer.

Bromide and iodide likewise cross the membrane with great rapidity, equilibrium being reached in 10 minutes and perhaps in a much shorter time. The manner of their distribution is, however, peculiar, in that at equilibrium the halide of the cells contains a higher proportion of bromide or iodide than does that of the serum. This peculiar distribution of bromide has been repeatedly observed (5). A rather elaborate explanation in which a chemical linkage of the bromide within the cells is assumed has been proposed by Hastings and van Dyke (5), but direct supporting evidence is lacking. Such a peculiar distribution is by no means confined to bromide and iodide; sulfanilamide and sulfapyridine likewise are regularly present in higher concentrations in cell water than in serum water (6). For the moment it is only possible to note the fact of such distributions, and to point out that there is no simple application of the membrane equilibrium theory which will serve to explain them.

Bromide distribution is evidently quite different from that of  $\text{Cl}^{38}$ , which is a true isotope of chloride and not just a somewhat similar substance. In many respects the body appears to treat them somewhat indifferently, but from this and from other instances (7) the cells may apparently distinguish between the two substances.

#### SUMMARY

1.  $\text{Cl}^{38}$  added to serum exchanges completely and very rapidly with all the chloride in the red blood cells.

2. Bromide and iodide also enter the red cells with great rapidity, equilibrium being established within less than 10 minutes.

3. At equilibrium the halide of the red cells contains proportionately more bromide or iodide than does that of serum.

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# THE RÔLE OF CARBON DIOXIDE IN THE GLUCOSE METABOLISM OF *TRYPANOSOMA LEWISI*

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The utilization of carbon dioxide by animal and plant cells and its relation to succinic acid formation has been the subject of extensive studies recently (1-7). It has been established through the work of Wood, Werkman, and coworkers (1, 2) with  $C^{13}$  in carbon dioxide as a tracer that, at least in the case of propionic acid bacteria, carboxylation of a 3-carbon atom compound takes place. Furthermore, it becomes highly probable in view of the work of Krebs and Eggleston (4) and others that the Wood-Werkman reaction, the carboxylation of pyruvic acid to oxalacetic acid, is the first step in the assimilation of carbon dioxide. The formation of succinic acid proceeds through the reduction of oxalacetate.

In previous reports (8, 9) on the glucose metabolism of the protozoon, *Trypanosoma lewisi*, it was shown that suspensions of this organism in buffered salt solutions containing glucose form under anaerobic conditions succinic acid, and that simultaneously carbon dioxide is assimilated. In the absence of bicarbonate, glucose is not metabolized by these trypanosomes under anaerobic conditions unless pyruvate is added. The data available at the time of our preliminary paper on this subject were not sufficient to allow a definite conclusion with regard to the mechanism whereby carbon dioxide is assimilated by suspensions of *Trypanosoma lewisi*. Since then additional information has been gathered which is given in this paper.

## EXPERIMENTAL

In all the experiments a suspension of washed trypanosomes obtained from the blood of infected rats was used. The density

varied between 1 and  $3 \times 10^8$  organisms per cc. They were suspended either in a mixture of 4 parts of a calcium- and magnesium-free Ringer's solution and 1 part of 0.15 M NaHCO<sub>3</sub>, or in 0.11 M phosphate buffer solution (pH 7.3). Both solutions contained 0.3 per cent glucose or glycerol. To study the effect of bicarbonate, mixtures of phosphate buffer and bicarbonate were also used. Oxygen consumption and carbon dioxide production or consumption were determined in the Warburg respirometer, with Dixon flasks when desirable. For the determination of acids produced, experiments were done in flasks containing about 100 cc. of trypanosome suspension. Glucose determinations were made by the Hagedorn-Jensen method. Pyruvic acid determinations were made according to Jowett. Succinic acid was determined by the method of Moyle. Lactic acid, formic acid, and volatile acid (acetic) were determined as described previously (8).

*Anaerobic Metabolism and Carbon Dioxide Effect*—In Table I the results are given of experiments demonstrating the utilization of bicarbonate and its effect on the rate of glucose metabolism. When bicarbonate was absent, the glucose disassimilated was very small. At the end of such an experiment the trypanosomes were motionless but they usually recovered when exposed to air during microscopic examination. Addition of a small amount of bicarbonate (7 micromoles per cc.) increased the rate of metabolism more than 5-fold and at the same time about 1 molecule of carbon dioxide disappeared per 3 molecules of glucose used. If the amount of bicarbonate was increased to 30 micromoles per cc., further increase in the rate of the glucose metabolism was observed and about 1 molecule of carbon dioxide was used per 2 molecules of glucose disassimilated. Further evidence for the assimilation of carbon dioxide was obtained in large scale experiments with Ringer's solution with bicarbonate. It is noteworthy that more than 2, *i.e.* 2.59, equivalents of fixed acids were formed from 1 molecule of glucose and that the total carbon represented by the metabolic products was more than the amount present in the glucose used. Corrected for undetermined acid, the total carbon was about 6.5 carbon atoms<sup>1</sup> per mole of glucose. It appears from

<sup>1</sup> Assuming that the undetermined acids contained 2 carbon atoms per equivalent of acid, then 0.19 carbon atom (3.6 per cent) was not determined; if they contained 3 carbon atoms, then 0.28 carbon atom was not determined.

the carbon balance that when large amounts of bicarbonate are present, 1 molecule of carbon dioxide was assimilated per 2 molecules of glucose. Furthermore, the moles of carbon dioxide assimilated are approximately equal to the moles of succinic acid produced and both are about equal to the excess over 2.00 of acid equivalents produced by 1 molecule of glucose.

Evidence for complete anaerobiosis and for a satisfactory account of all metabolites was demonstrated by the oxidation-reduction balance. The oxidation value of the succinic (520 micromoles) and pyruvic acids (605 micromoles) produced was

TABLE I  
*Anaerobic Carbon Dioxide and Pyruvate Effects\**

Medium	Gas mixture	Time	CO <sub>2</sub> used	Glucose used	CO <sub>2</sub> Glucose
		min.	micro-moles per 10 <sup>10</sup> trypanosomes	micro-moles per 10 <sup>10</sup> trypanosomes	
Phosphate buffer + glucose	N <sub>2</sub>	90		27	
		180		83	
Phosphate buffer + glucose + 0.6 mg. NaHCO <sub>3</sub> per cc.	"	90	66	211	0.31
		180	102	365	0.28
Ringer's solution + bicarbonate	" (95%)	90	157	253	0.62
	CO <sub>2</sub> (5%)	180	242	425	0.57
Phosphate buffer + glucose + 1 mg. pyruvate per cc.	N <sub>2</sub>	90		120	
		180		254	

\* The values given are averages of several experiments; the glucose used in the absence of pyruvate or bicarbonate was always much less than in their presence.

1125 and that of the carbon dioxide used (about 500 micromoles) was 1000 (*cf.* Wood and Werkman (1)).

*Anaerobic Pyruvate Effect*—Although many substances were tested, pyruvate and oxalacetate were the only two substances which also supported to some extent the anaerobic glucose metabolism of these organisms. If a small amount of pyruvate was added to a carbon dioxide-free medium containing glucose, the rate of glucose disassimilation was about two-thirds of that found in the presence of carbon dioxide (*cf.* Table I) but the products of the metabolism were different, indicating that pyruvate is not an



equivalent of carbon dioxide in its effect on the glucose disassimilation (*cf.* Table II). Pyruvate disappeared in these experiments, while it was produced when bicarbonate was added to the medium. Succinic acid was not produced in appreciable quantities and lactic acid and a small amount of acetic acid were formed. Thus the effect of pyruvate is different from that of bicarbonate and it is not probable that the pyruvate effect can be attributed to decar-

TABLE II  
*Anaerobic Metabolism*

Medium	Acids produced	Micro-equivalents per 10 <sup>10</sup> trypanosomes	Equivalents per mole glucose	C atoms per mole glucose	Acid equivalent	Remarks
					<i>per cent</i>	
Ringer's solution + bicarbonate + glucose (95% N <sub>2</sub> + 5% CO <sub>2</sub> )	Succinic	1040	1.00	2.00	38.8	Glucose used, 1040 micro-moles per 10 <sup>10</sup> trypanosomes; duration, 300 min.
	Lactic	789	0.76	2.28	29.3	
	Pyruvic	605	0.58	1.74	22.5	
	Acetic	155	0.15	0.30	5.8	
	Formic	<10				
Total . . . . .		2690*	2.59*	6.32	96.4	
Phosphate buffer + pyruvate + glucose (N <sub>2</sub> )	Lactic	1910	1.87	5.61	95.5	Glucose used, 1020 micro-moles per 10 <sup>10</sup> trypanosomes; duration, 240 min.
	Pyruvic†	-304	-0.30	-0.90	-15.3	
	Acetic	240	0.24	0.48	12.3	
Total . . . . .		2000*	1.96*	5.19	92.5	

\* Determined by titration.

† Used instead of produced.

boxylation of pyruvic acid and simultaneous utilization of carbon dioxide. Pyruvate added to a trypanosome emulsion was not attacked in the absence of glucose under either aerobic or anaerobic conditions. Succinic acid was not formed from pyruvate and bicarbonate in the absence of glucose.

The oxalacetic acid effect could be attributed to the decomposition of oxalacetate to pyruvate and carbon dioxide, which was found to take place in control experiments.

*Aerobic Metabolism*—Under conditions otherwise similar to those described previously but in the presence of oxygen, carbon dioxide was produced instead of being used. The ratio of total acid equivalents (fixed) produced to the moles of glucose disassimilated was approximately 2. There is a striking difference in the relative proportions of the various acids produced. Only a small amount of succinic acid was formed but the amount of acetic acid produced (50 per cent of the total acid equivalents) was about the same as the equivalents of succinic acid produced under anaerobic conditions. The often discussed correlation between succinic acid formation and acetic acid formation obviously also exists in the

TABLE III  
*Aerobic Glucose Metabolism*

Medium, Ringer's solution + bicarbonate + glucose; gas mixture, 95 per cent O<sub>2</sub> + 5 per cent CO<sub>2</sub>; duration, 240 minutes; glucose used, 394 micromoles per 10<sup>10</sup> trypanosomes.

Acid produced	Micro-equivalents per 10 <sup>10</sup> trypanosomes	Equivalents per mole glucose	C atoms per mole glucose	Acid equivalent
				<i>per cent</i>
Acetic .....	376	0.95	1.90	45.5
Succinic .....	82	0.20	0.40	9.9
Lactic .....	158	0.40	1.20	19.1
Pyruvic .....	153	0.39	1.17	18.5
Formic .....	15	0.04	0.04	1.8
Total .....	828*	2.1*	4.71	94.8

\* Determined by titration.

present case. The other acids produced were lactic and pyruvic acids (*cf.* Table III).

As reported in previous communications, about 1 mole of oxygen is used per mole of glucose and approximately 1 mole of carbon dioxide was produced per mole of glucose, the respiratory quotient being about 1.0. In contrast to anaerobic conditions, glycerol was well disassimilated under aerobic conditions. However, no fixed acid was produced and the glycerol was oxidized quantitatively to carbon dioxide and water. The respiratory quotient was 0.85, which is in agreement with the theory (0.86) (*cf.* Table IV).

*Aerobic Carbon Dioxide Effect*—Although carbon dioxide is a product of both the aerobic glucose and glycerol metabolism, and

apparently is not assimilated under these conditions, carbon dioxide possesses, nevertheless, what appears to be an activating effect under aerobic conditions. This effect was measured in terms of

TABLE IV  
*Aerobic Glycerol Metabolism*

Medium, phosphate buffer + glycerol with and without added bicarbonate (air); duration, 180 minutes.

	O <sub>2</sub> used	CO <sub>2</sub> produced	R. Q.	Activity
	<i>micromoles per 10<sup>10</sup> trypanosomes</i>	<i>micromoles per 10<sup>10</sup> trypanosomes</i>		
With NaHCO <sub>3</sub> (0.03 M).....	3240	2760	0.85	Very active
Without.....	915	788	0.86	Inactive

TABLE V  
*Aerobic Carbon Dioxide Effect*

Substrate, glucose; duration, 30 min.

Medium	Gas mixture	Glucose used
		<i>micromoles per 10<sup>10</sup> trypanosomes</i>
Phosphate buffer	O <sub>2</sub>	0
Ringer's solution + bicarbonate	"(95%) CO <sub>2</sub> (5%)	86

Medium, phosphate buffer + glycerol (air)

Time	O <sub>2</sub> used	
	Control	With NaHCO <sub>3</sub> (0.03 M)
<i>min.</i>	<i>c.mm.</i>	<i>c.mm.</i>
10	1.5	37
35	4.5	68
60	12.0	100

increased rate of oxygen consumption upon the addition of bicarbonate to a suspension of trypanosomes in carbon dioxide-free phosphate buffer with glucose or glycerol as the substrate (*cf.* Tables IV and V).

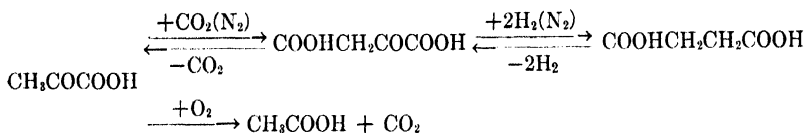
The carbon dioxide effect was observed clearly with suspensions of trypanosomes in a carbon dioxide-free phosphate buffer in which the trypanosomes had become motionless ("resting") because of lack of substrate. If glycerol was added, and oxygen was bubbled through this suspension, most of the trypanosomes remained motionless for about an hour or more. If, on the other hand, oxygen containing 5 per cent carbon dioxide was bubbled through the suspension, the trypanosomes revived quickly and became very active in less than 5 minutes. A similar effect was found with glucose as the substrate but was less marked as the trypanosomes revived even when pure oxygen was bubbled through the suspension. Pyruvate had no influence on this phenomenon.

#### DISCUSSION

The experimental evidence indicates that under anaerobic conditions (nitrogen, and also hydrogen and carbon monoxide) carbon dioxide is assimilated by *Trypanosoma lewisi* suspended in buffered glucose solution. This may take place either by direct reduction and polymerization as suggested previously or by condensation of carbon dioxide with another compound, followed by the reduction of the new compound. Propionic acid bacteria also assimilate carbon dioxide and produce succinic acid, but at variance with *Trypanosoma lewisi* they are able to do so when glycerol is the substrate. The fact that for each carbon dioxide molecule used a succinic acid molecule is formed supports the assumption that condensation resulting in carboxylation takes place, although assimilation of carbon dioxide by reduction and polymerization and the formation of succinic acid according to Toennissen and Brinkmann (10) is also compatible with the analytical results. However, if carbon dioxide were reduced to formaldehyde, formic acid should be an intermediate. But formic acid was not reduced when added to the system. Increase of succinic acid formation through the addition of acetic acid or pyruvic acid could not be demonstrated.

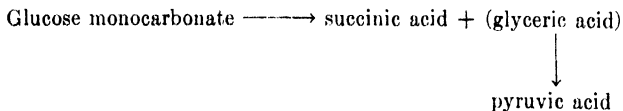
The correlation between succinic acid formation with carbon dioxide utilization under anaerobic conditions and acetic acid formation with carbon dioxide production under aerobic conditions suggest that there is a common source for these products. This

could be pyruvic acid according to the following scheme involving the Wood-Werkman reaction of carboxylation.



Thus the absence of succinic acid under aerobic conditions may be explained by assuming that since oxalacetic acid cannot be reduced under aerobic conditions in this system, it does not accumulate but decomposes through pyruvic acid into carbon dioxide and acetic acid.

There are, however, two experimental facts which cannot be reconciled with the above equation involving the Wood-Werkman reaction; first, pyruvic acid does not undergo any change if bicarbonate is present, and second, carbon dioxide is needed for the glucose and especially for the glycerol disassimilation under aerobic conditions. In the case of glycerol there was no evidence that either pyruvic acid or succinic acid is formed as an intermediate or final product. Thus, the aerobic carbon dioxide effect seems to be due to a reaction different from that ordinarily called carboxylation. It was suggested previously (9) that under aerobic conditions the bicarbonate-formate system may act as a hydrogen carrier. However, the addition of formic acid does not have any effect on the aerobic glycerol metabolism. The possibility that the catalytic action of carbon dioxide is due to carbonate ester formation of both glucose and glycerol, we believe, deserves consideration. Succinic acid formation could then result from an intermolecular oxidation-reduction and the rearrangement of glucose monocarbonate.



In the aerobic oxidation of glycerol, it is conceivable that the ring compound, glycerol carbonate, is oxidized simultaneously at various carbon atoms without accumulation of oxidized 2- or 3-carbon atom derivatives. The experimental evidence on which

this assumption is based is that glycerol is not an adequate substrate unless bicarbonate is present. The evidence, although indirect, gains in significance by the fact that not even traces of intermediates of glycerol oxidation could be found. It may be of interest to note in this connection that while fluoride inhibited the anaerobic metabolism, direct evidence was not found for phosphorylation of glucose and glycerol by trypanosomes. Carbonate formation may have a rôle similar to phosphorylation in the activation of certain substrates or intermediates.

#### SUMMARY

Carbon dioxide activates the anaerobic and aerobic glucose and the aerobic glycerol disassimilation.

In the anaerobic glucose disassimilation, succinic, lactic, pyruvic, and acetic acids are formed, and carbon dioxide is used. The amount of assimilated carbon dioxide is equivalent to the amount of succinic acid formed.

Pyruvate also activates the anaerobic glucose disassimilation but succinic acid is not formed. Pyruvate does not activate the aerobic glucose and glycerol disassimilation.

Under aerobic conditions about as much acetic acid is produced as succinic acid equivalents under anaerobic conditions. In addition small amounts of succinic acid were found and some lactic and pyruvic acids.

Glycerol is disassimilated under aerobic conditions only. The products of metabolism are carbon dioxide and water.

The aerobic metabolism of glycerol as well as that of glucose is activated by bicarbonate. This can be demonstrated strikingly when "resting" trypanosomes are used.

*Trypanosoma lewisi* does not form succinic acid from pyruvate and bicarbonate and the former is not decarboxylated. The possibility has been suggested that carbon dioxide utilization under anaerobic conditions and carbon dioxide activation of the metabolism under aerobic conditions involve glucose carbonate and glycerol carbonate formation.

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**THE REPLACEABILITY OF *dl*-METHIONINE IN THE DIET  
OF THE ALBINO RAT WITH *dl*-METHIONINE SULFONE  
AND *dl*-METHIONINE METHYLSULFONIUM  
CHLORIDE\***

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In a previous experiment, *dl*-methionine sulfoxide, an oxidation product of *dl*-methionine, was found capable of replacing *dl*-methionine in the diet of the albino rat (1). The results of that experiment suggested that *dl*-methionine sulfoxide could possibly play an intermediate rôle in the metabolism of methionine. Since then another oxidation product of *dl*-methionine, *dl*-methionine sulfone, has been prepared by Toennies and Kolb (2). As this compound is in a more advanced state of oxidation than the sulfoxide, it is of interest to find out whether the sulfone can be utilized for growth. Investigations on the rôle of several oxides of cystine in the intermediary metabolism of cystine suggest that increase in the state of oxidation of the —SH means decrease in its reversible reduction capacity (3, 4). Growth curves were obtained of albino rats fed *l*-cystine disulfoxide, S-(guanyltio)cysteine + 2HCl, which hydrolyzes to *l*-cysteinesulfenic acid, and *l*-cysteinesulfinic acid. *l*-Cystine disulfoxide proved capable of replacing *l*-cystine in the diet, but in growth promotion, 3 molecules of the disulfoxide are apparently the equivalent of only 1 molecule of *l*-cystine. The *l*-cysteinesulfenic acid gave rise to a slight but definite increase in growth, while the *l*-cysteinesulfinic acid produced no growth.

The other compound reported in this paper, *dl*-methionine methylsulfonium chloride, prepared by Toennies,<sup>1</sup> is of interest because of its two methyl groups and raises the question as to

\* Aided by a grant from the Robert McNeil Fellowship.

<sup>1</sup> Toennies, G., unpublished data; see also (5).



whether the rat can make use of such a compound for growth in the absence of an adequate supply of methionine in the diet.

### *Preparation of Compounds*

The compounds employed were *dl*-methionine (University of Illinois), *l*-cystine (Merck), *dl*-methionine sulfone (2), *dl*-methionine methylsulfonium chloride, prepared as described by Toennies.<sup>1</sup> These compounds were over 99 per cent pure as shown by analysis. Arachin was isolated from peanut meal<sup>2</sup> by the method of Johns and Jones (6), acetone being used as the drying agent instead of alcohol and ether. The arachin used in the present experiment was analyzed for methionine by the method of Kassell and Brand<sup>3</sup> (7) and contained  $0.47 \pm 0.04$  per cent methionine by the homocysteine titration and  $1.12 \pm 0.14$  per cent cystine. These determinations were carried out on dried samples; the arachin when dried to a constant weight at 100° lost approximately 7.5 per cent and had 0.86 per cent ash.

### EXPERIMENTAL

Albino rats, Wistar strain, 25 days old were used as experimental animals. They were kept on a methionine-deficient diet, a modification of the cystine-deficient diet of Dyer and du Vigneaud (8) with the following percentage composition: milk vitamin concentrate 16, arachin (as the basal protein) 15, dextrin 24, sucrose 15, Osborne and Mendel (9) salt mixture 4, agar 2, lard 19, and cod liver oil 5, fed *ad libitum*; each rat received 100 mg. of the Harris vitamin B complex daily. Sufficient dry mixture was made at the beginning of the experiment to last through the entire period. From this, the fresh basal diet was prepared every 3 days. All food was kept in the refrigerator. The compounds, with the exception of cystine, were fed individually in an approximately 50 per cent sucrose solution, the daily dose being contained in 0.2 cc. of the sucrose solution. The rats took the liquid from a pipette. The control animals received the same amount of the sucrose solution without the added compounds. New solutions were made on the 5th and the 12th days of the test period, and kept in the refrigerator at -10°. Since the sulfonium chloride could not be

<sup>2</sup> I wish to thank the Planters Peanut Company for the peanut meal.

<sup>3</sup> I am indebted to Mr. Thomas P. Callan for these determinations.

prepared in crystalline form, it would not mix with butter, as in the technique employed in previous experiments. Therefore sucrose solutions were used. Cystine, however, being insoluble, was given individually in small pieces of butter. The butter mixtures were made once a week, all instruments being kept on ice during the mixing, and were kept in individual covered glass dishes at 0°. A control group was not run for the butter, as control groups on the

TABLE I  
*Daily Basal Food Consumption per Rat in Gm. Averaged over a 2 Day Period*

Days	Group A, unsupple- mented control	Group B	Group C	Group D	Group E
		Unsupplemented			
1- 2	5.8	5.6	5.3	5.5	5.5
3- 4	5.5	5.2	4.9	5.2	5.2
5- 6	4.9	4.5	4.0	4.5	4.2
7- 8	5.2	4.4	4.1	4.2	4.3
Daily supplement					
		8.03 mg. <i>dl</i> -methio- nine methyl- sulfonium chloride	6.0 mg. <i>dl</i> -methio- nine	7.29 mg. <i>dl</i> -methio- nine sulfone	4.83 mg. <i>l</i> -cystine
9-10	4.2	4.0	5.4	4.1	4.4
11-12	5.7	4.6	5.4	4.8	4.7
13-14	5.3	5.0	5.2	5.2	4.4
15-16	6.2	5.8	4.7	4.9	4.7
17-18	5.5	5.3	4.3	4.5	3.1
19-20	4.3	4.3	4.0	3.6	2.9
21-22	4.5	4.7	4.5	4.1	3.5
23-24	4.5	5.1	5.0	3.7	3.3
9-24 (Total)	80.4	77.6	77.0	69.8	62.0

arachin basal diet in previous experiments (4, 1) received butter, and showed no apparent effect on the growth curves. The animals were maintained on a normal diet for 6 days and on a basal diet for 8 days, at the end of which the basal diet was supplemented with the various compounds to be studied for a period of 16 days. The rats were weighed every other day and the average weight of the group plotted. The approximate amount of the basal diet consumed per rat each day was determined by weighing daily the basal

food given each group and the residual food, and dividing by the number of animals in the group (Table I).

In the experiment reported, twenty-four rats from three litters, one litter 26 days old, the other two 25 days old, were divided into five comparable groups. Four groups contained three males and two females each; the control group contained two males and two females. After 8 days on the methionine-deficient diet, the

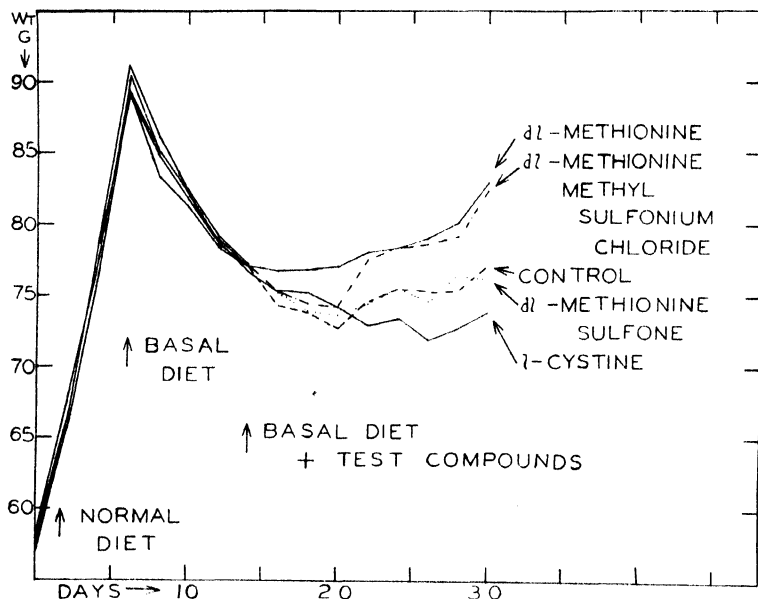


FIG. 1. Growth curves showing the replaceability of *dl*-methionine in the diet of the albino rat with *dl*-methionine sulfone and *dl*-methionine methylsulfonium chloride. The casein of Dyer and du Vigneaud's basal diet was replaced by arachin.

diet of Group A, the control group, was left unsupplemented except for the sucrose solution; that of Group B was supplemented daily for 16 days with 8.03 mg. of *dl*-methionine methylsulfonium chloride; that of Group C with 6.0 mg. of *dl*-methionine; that of Group D with 7.29 mg. of *dl*-methionine sulfone, the amount of the sulfone fed being doubled the last 4 days; that of Group E with 4.83 mg. of cystine. These were amounts containing the sulfur equivalent of 6.0 mg. of *dl*-methionine. Fig. 1 shows the results

obtained. After 6 days the curve of the group receiving the sulfonium salt rose to the height of the curve of the methionine group and continued with it. The sulfone curve followed that of the control group, while the cystine curve fell below that of the control.

#### DISCUSSION

Previous work (1), carried out under the same conditions as the present experiment, showed that methionine sulfoxide supports growth to the same extent as its parent substance, methionine. However, the conversion of methionine to the sulfone seems to destroy completely its growth-supporting properties. Increase in the state of oxidation of the sulfur evidently increases the irreversibility of the reaction, as suggested by similar experiments with several oxides of cystine mentioned in the introduction to this paper (3, 4). During the last 4 days of the experiment the amount of the sulfone fed was doubled but the curve continued to follow that of the control group. This indicates that a larger amount of the compound would not produce growth and that the sulfone was not toxic, although the rats showed a marked dislike for it.

The rats were able to utilize the sulfonium salt, *dl*-methionine methylsulfonium chloride, for growth in this experiment when a methionine deficiency was present. However, 6 days elapsed before the growth curve became practically identical with that of the methionine. At first the curve fell with that of the control group but on the 6th day it rose suddenly to the level of that of the methionine group. The rise was so steep that it suggests that the rats may have developed some special mechanism for taking care of the extra methyl group and by that means converted the sulfonium compound into methionine. The extra methyl group freed by this reaction might possibly have a sparing effect on methionine in the formation of choline.

The possible metabolic significance of sulfonium reactions has been discussed by Toennies (5). He suggests a hypothesis for the mechanism of the conversion of methionine to cystine based on the capacity of methionine to form sulfonium derivatives and the participation of the hydroxyamino acids. The present experiment gives evidence that the rat can metabolize a sulfonium salt.

Cystine was fed to determine the degree of methionine deficiency of the arachin used in the basal diet, since in previous experiments of this series, when arachin was used as the methionine-

deficient protein, cystine was not fed. The cystine curve fell even below that of the control group. The fact that there was no growth was to be expected from the experiments of White and Beach (10) who demonstrated that *l*-cystine is incapable of producing growth of the albino rat when added to a 15 per cent arachin diet. Therefore, the present diet must have contained less than 0.1 per cent methionine, a figure which is approximately the minimum amount of methionine that can be supplemented by *l*-cystine (11). The fact that the curve was so low seems to suggest a slight toxicity which cannot be explained at present. It is probably not due to a choline deficiency, since the 16 per cent milk vitamin concentrate used in the diet would furnish sufficient choline (12). Autopsies on the cystine-fed rats showed, on microscopic examination, a few scattered intertubular hemorrhages of the kidneys not present in the control animals.<sup>4</sup>

#### SUMMARY

1. *dl*-Methionine sulfone did not replace methionine in the diet of the albino rat under the conditions of the present experiment. The sulfone, however, did not appear to be toxic.

2. *dl*-Methionine methylsulfonium chloride replaced methionine in the diet of the albino rat under the conditions of this experiment, indicating that a mechanism for the metabolism of a sulfonium salt is available to the animal.

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<sup>4</sup> I am indebted to Elizabeth Stavers Barden and Dr. Roland S. Aronson for this report.

# THE INFLUENCE OF THE METABOLISM OF HUMAN ERYTHROCYTES ON THEIR POTASSIUM CONTENT\*

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Davson (2) has recently reviewed the subject of the cation permeability of erythrocytes. Although many environmental changes have been shown to affect the exchange of sodium or potassium or both, little insight has been gained concerning the factors responsible for the so called "normal impermeability" of the red cell membrane to these ions. The means by which the normal distribution is acquired is likewise obscure.

Recent work by Eisenman *et al.* (3) and Dean *et al.* (4) with radioactive cations has indicated that there is a slow exchange of potassium across the membranes of the human, rat, and rabbit erythrocytes, the rate of which varies with the species. Cohn and Cohn (5) have shown that radioactive sodium enters the erythrocyte of the dog *in vivo*. These results suggest that there is a dynamic equilibrium between the cations in the erythrocytes and those in the surrounding medium.

Evidence to be presented in this paper indicates that in human blood the potassium and sodium distribution between the cells and the extracellular medium is greatly influenced by the metabolism of the erythrocytes, and that the factors operative in the original accumulation of potassium within the cells are probably responsible for the maintenance of the concentration gradient normally observed.

## Methods

All observations reported here were made on human erythrocytes. Both fresh and preserved bloods were used. Blood was

\* A preliminary account of this work has been reported (1).

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preserved according to the technique already described (6). Unless otherwise specified, sodium citrate was used as the anti-coagulant (2 parts of 3.2 per cent  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$  to 23 parts of blood, designated as the citrate-blood mixture).

In certain experiments the citrated blood was diluted with an isosmotic glucose solution (2 parts of citrate, 13 parts of 5.4 per cent glucose, and 10 parts of blood, referred to as the citrate-glucose-blood mixture). This mixture retards hemolysis much longer than does citrate alone (6). Aseptic precautions were observed throughout.

Generally, the cells of a given volume of blood mixture were analyzed, although a few plasma analyses are reported. 1 or 2 ml. of blood mixture were centrifuged and the supernatant fluid carefully removed. The sides of the tube were washed with 5 ml. of a 10 per cent sucrose solution, care being taken not to disturb the cells. The material was again centrifuged and the supernatant fluid removed as before. The cells were finally washed into a silica crucible and ashed with the aid of sulfuric acid; the ash was dissolved in hot water, made up to a convenient volume, and filtered. Suitable aliquots were taken for potassium analyses by the method of Harris (7) and for sodium by the method of Salit (8). With this procedure analyses are carried out on a constant number of cells and no correction need be applied for hematocrit change. (Except as indicated, no correction is made for hemolysis, which in general was less than 2 per cent.)

The procedure was checked against two possible sources of error. It has been shown that suspension of erythrocytes in a non-electrolyte medium results in a potassium loss (9, 10). The washing procedure as here described, however, induced no such loss. Secondly, since the cells were not suspended in the washing medium, the possibility of error arising from potassium remaining within the interstices of the cells was checked and found to be negligible. In checking this point isotonic potassium chloride was added to one of duplicate blood samples and the potassium content of the cells of both determined as above. The entire procedure is accurate within 2 per cent.

Whole blood phosphates were determined by the method of Fiske and Subbarow (11), glucose by the methods of Shaffer and Somogyi (12) and Somogyi (13), and plasma hemoglobin by our adaptation of the method of Wu (14). The Van Allen pipette

was employed for hematocrit determinations, no diluent being used.

*Potassium Loss from Human Erythrocytes during Storage at 2-5°*—When blood is stored at refrigerator temperatures, potassium diffuses from the red cell (15-17) and sodium enters (18, 19).<sup>1</sup> Neither the use of various preservatives nor the maintenance of a normal calcium concentration has been found to prevent this loss (15, 20),<sup>1</sup> although modifications in the extent of loss have been reported (19).<sup>1</sup>

This potassium loss has been taken as evidence of erythrocyte degeneration (21). What appears to have escaped attention is the first order form of the curve (Fig. 1). The experiments here reported were chosen because hemolysis was minimal in the 10 per cent sucrose and 5.4 per cent glucose solutions employed. Essentially the same form of curve was obtained with normal electrolyte concentrations. In the graph (Fig. 1) the ordinate is expressed as mm of potassium in the plasma associated with 100 ml. of normal sized cells, thus correcting for hematocrit changes and differences in dilution. It will be observed that the curves can be divided into two phases, the first in which the rate of diffusion of potassium falls progressively with time, equilibrium being reached in 15 to 20 days, and the second during which the diffusion rate is mostly constant with time. (As given here the time intervals over the first phase are too long for an accurate analysis of the curve. However, observations over shorter periods made in this laboratory and elsewhere (17) are in agreement.)

The form of the first phase indicates that the factor responsible for this diffusion of potassium is immediately operative, and that during this period the permeability remains essentially unchanged. To verify this the equation  $dZ/dt = kA(C - Z)$  may be used, where  $Z$  is the amount diffusing in any time  $t$ ,  $A$  the surface area of the cells,  $C$  the total diffusion to equilibrium, and  $k$  a form of permeability constant.<sup>2</sup> Since the experimental values are

<sup>1</sup> Also DeGowin, E. L., Harris, J. E., Bell, J., and Hardin, R. B., unpublished results.

<sup>2</sup> Exact conformity of the data to this equation would be expected only if the cell volume remained constant, which, strictly speaking, is not the case. However, the sodium entering the cell balances to a large extent the potassium leaving, so that this expression can serve as a first approximation, which suffices for our purposes.



referred to a standard volume and number of cells, the surface area may be ignored for the purpose of this calculation. With the 15 day value as the equilibrium figure, the constant,  $k$ , was found to be as given in Fig. 1 at the 5 and 10 day periods. The values agree sufficiently well to indicate that the postulation made above is correct; *i.e.*, the diffusion begins immediately and does not represent a progressive cellular deterioration. The permeability of the erythrocytes to potassium was the same in both preserva-

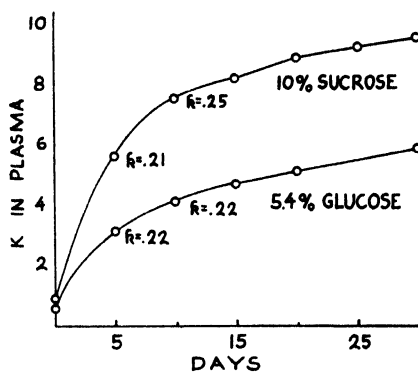


FIG. 1

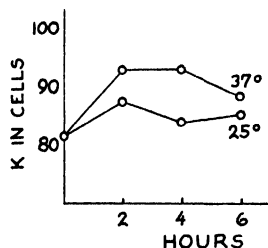


FIG. 2

FIG. 1. The increase in plasma potassium concentration during storage of human blood at 2-5°. Ordinate, mm of potassium in the plasma associated with 100 ml. of normal sized cells.  $k$  = permeability constant.

FIG. 2. The effect on the potassium content of the cells of raising the temperature of a preserved citrate-blood mixture. The blood mixture had been stored 5 days at 2-5°. Ordinate, mg. of potassium in the cells of 100 ml. of blood mixture.

tives, although the total amount diffusing was much greater in the sucrose solution. The difference may be attributed to the fact that the cells swell in glucose solution, thus decreasing the cellular concentration of potassium.

*Migration of Potassium from Plasma to Cells When Stored Blood Is Placed at 25° and 37°*—In attempting to explain the fact that refrigeration induced an immediate diffusion of cation across the cell membrane, it seemed to us that the lowered metabolism might be the factor in question and that raising the metabolism by elevating the temperature might reverse this effect. It was found

that not only did the potassium cease coming from the cells when the temperature was raised, but that it reentered against a concentration gradient. Typical results are plotted in Fig. 2. Here, as with the rest of the data to be presented, potassium values are expressed as mg. in the cells of 100 ml. of blood mixture. A 5 day-old citrate-blood mixture was removed from the refrigerator and samples placed at 25° and at 37°. At both temperatures the potassium migrated from the plasma to the cells, the effect being greater at 37°. It will be noticed that there was a tendency for the potassium content of the cells to fall off at 6 hours. This tendency was also seen in other experiments, although the drop generally occurred later.

*Effect of Glucose on Penetration of Potassium into Human Erythrocytes*—Since glucose was being depleted by glycolysis, the effect of adding this substance when the temperature was raised was next determined. (The total reducing substances in stored blood vary but have usually diminished by more than one-half after 5 days and reached a minimum at 10 days.) Typical results of such experiments are plotted in Fig. 3. Here a citrate-blood mixture was stored at refrigerator temperatures, samples being removed at the stated intervals and placed at 25° or 37°. In certain cases glucose, as a solid, was added to these samples in sufficient quantity to raise the concentration to approximately 400 mg. per cent. The figures in parentheses represent the pH at the end of each experiment. It will be observed that the presence of glucose had a pronounced effect on the amount of potassium which reentered the cells after storage. Moreover, when glucose was added, the potassium content of the cells continued to rise for at least 24 hours, although it showed a tendency to level off at that time. However, the utilization of glucose was not necessary for some accumulation of potassium against a concentration gradient. For example, in the 5 day-old blood to which no glucose had been added, this substance was depleted after 3 hours (probably before) at 37°; yet the potassium continued to increase for at least another 8 hours. Sometime after 11 hours potassium began to diffuse from the cell, resulting in a low value at 24 hours.

Since potassium may reenter the cell when glucose is not being metabolized, no relationship between the amount of potassium

reentering and the glycolytic activity would be expected. Qualitatively, however, it is obvious that as this activity diminishes the amount of potassium reentering also decreases. If a quantitative relationship existed, it would be found at the beginning of the experiment, when the rate of entrance of potassium is highest.

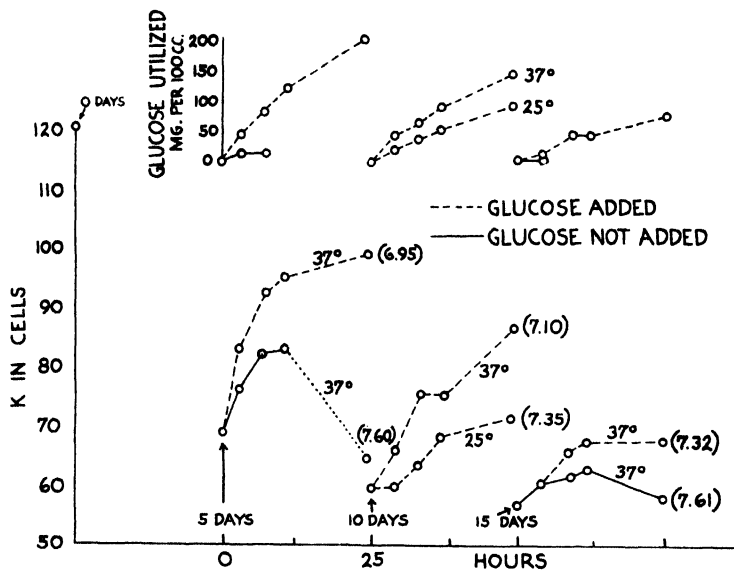


FIG. 3. The effect of the addition of glucose on the increase in the potassium content of the cells of a citrate-blood mixture placed at 37° or 25° after storage in the refrigerator. Ordinate, mg. of potassium in the cells of 100 ml. of mixture. The abscissa is not continuous but is divided into 25 hour intervals, corresponding to the total periods of observations on the blood mixtures removed from the refrigerator after 5, 10, and 15 days of storage. The 15 day-old mixture showed 4.0 per cent hemolysis when removed from the refrigerator and 6.8 per cent after 25 hours at 37° with no glucose added. The figures in parentheses represent the pH at the end of each experiment.

The ratio of the moles of glucose used per mole of potassium reentering was computed for five bloods of different ages after 3 to 4 hours at 37°. The calculated values were 0.79, 0.57, 0.64, 1.24, and 0.55, four of which demonstrated certain consistency. (The high value is that computed from a 10 day-old sample at 37° (Fig. 3) which showed a very irregular curve.)

When glucose was added to a citrate-blood mixture that had been stored 20 days and placed at  $37^{\circ}$ , little or no glycolysis occurred. There was also little change in the potassium content of the erythrocytes.

It should be pointed out that during storage at refrigerator temperatures there is a marked increase in the inorganic phosphate of citrate-blood mixtures beginning about the 10th day of storage.

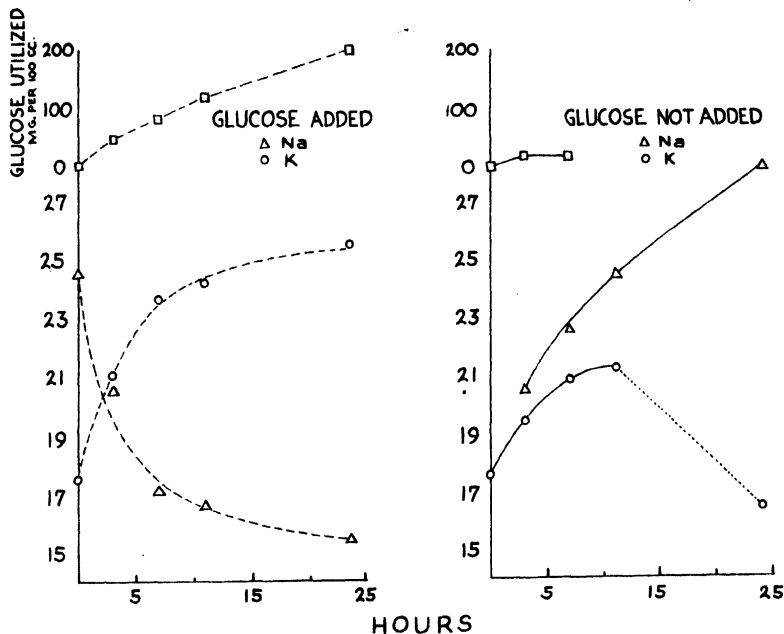


FIG. 4. The relation of the sodium to the potassium migration in a citrate-blood mixture after storage for 5 days at  $2-5^{\circ}$ . Ordinate, mm of cation in the cells of 1000 ml. of mixture.  $T = 37^{\circ}$ .

The values for one experiment (Fig. 3) were 2.76, 2.67, 6.13, and 13.5 mg. of P per 100 ml. of blood mixture after 0, 5, 10, and 15 days of storage respectively. This increase has been shown to be entirely at the expense of the organic acid-soluble fraction (20). The inorganic phosphorus thus formed remains mostly within the cells.

*Relation to Sodium Diffusion*—As has been mentioned previously, sodium enters human erythrocytes during blood storage, the ki-

netics of the diffusion being similar to the kinetics of the potassium loss. The exchange is not necessarily equivalent but depends to a large extent upon the concentrations of these ions in the extracellular medium. Nevertheless, stored human erythrocytes contain sufficient sodium to be readily adapted to the system of analysis employed for potassium.

From observations that the hematocrit values remained constant it had been expected that as potassium reentered the cell when preserved blood was placed at 37° sodium would diffuse out. In Fig. 4 it can be seen that this was true to a certain extent. In this experiment glucose was added to one portion of a 5 day-old citrate-blood mixture and both samples were placed at 37°. When glucose was added, sodium diffused from the cell as potassium entered, the exchange being approximately equivalent. The migration in both instances was against a concentration gradient. Similar observations were made at 25°. When the only available glucose was that which remained after storage, a different picture was seen. A decrease in the sodium content of the cell was seen at 3 hours, after which it showed a steady increase. (No 0 hour sodium analysis was made, but it may be assumed to be the same as in the sample to which the glucose was added.) Thus, for the period between 3 and 12 hours potassium and sodium were migrating in the same direction; *i.e.*, into the cell, the first against and the second with a concentration gradient. The same process was noted in a 15 day-old citrate-blood mixture to which no glucose was added (Fig. 5).

This phenomenon will require more study, but it appears that while the erythrocytes which have been stored are actively glycolyzing, either at 25° or at 37°, potassium enters the cell and sodium leaves it. On the other hand, when no glucose is present, potassium may still reenter the cell against a concentration gradient, whereas sodium appears to move with the gradient into the cell. It should be emphasized that the equivalence of exchange is not always observed and does not appear to be a necessary feature. Since the exchanges of cations during storage are frequently not equivalent, this finding is not surprising.

*Effect of Glucose on Loss of Potassium from Erythrocytes of Fresh Human Blood*—It has been shown repeatedly that red cells, suspended in an isotonic solution of electrolytes or in their own serum

or plasma, maintain their potassium content for a number of hours at room or body temperature. Eventually, however, this ion diffuses from the cells. In view of the fact that glycolysis exerts such an influence on the recovery of potassium after storage, it seemed logical that the appearance of a potassium loss from the cells of fresh blood after a number of hours might be associated with the disappearance of glucose and the cessation of active glycolysis.

Glucose, as a solid, was added to one sample of a citrate-blood mixture, the other serving as a control, and the glucose and cellular

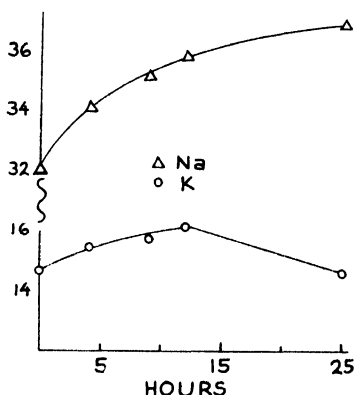


FIG. 5. The relation of the sodium to the potassium migration in a citrate-blood mixture after storage for 15 days at refrigerator temperatures. Ordinate, mm of cation in the cells of 1000 ml. of mixture.  $T = 37^{\circ}$ .

potassium were followed in both for a number of hours at  $25^{\circ}$  or  $37^{\circ}$ . The results are given in Table I. It can be seen that, for the first 10 to 12 hours at least, the potassium content of the red cells was maintained very well whether or not glucose was added. At 24 hours the cells of blood to which no glucose had been added showed a loss of potassium. In Experiment 2c, glucose was added to the control after 30 hours at  $37^{\circ}$  in an attempt to incite glycolysis. The carbohydrate was utilized to a certain extent but apparently not in sufficient quantity to increase the potassium content of the cells, a finding which is in accordance with the results of the experiments with 20 day-old citrate-blood mixtures.

On the other hand when glucose was added in the early part of

the experiment (Experiments 1b, 2a, 2b), the erythrocytes showed no loss of potassium for the remainder of the observations. It is interesting to note that this was true at both 25° and 37°, although the glycolytic rate at the latter temperature was twice as great.

TABLE I

*Effect of Glucose on Potassium Loss from Cells of Fresh Citrate-Blood Mixture at 25° and 37°*

The values are expressed in mg.

Time of observation hrs.	Potassium content in cells of 100 ml. blood		Glucose utilized per 100 ml. blood			
	Experiment 1a, 37°	Experiment 1b, 37°	Experiment 1a, 37°	Experiment 1b, 37°		
0	136	136				
2	137	137	16	16		
5	138	138*	34	34*		
7	136	136	52	52		
10	137	136	53	82		
24	128	141		204		
	Experiment 2a, † 25°	Experiment 2b, † 37°	Experiment 2c, 37°	Experiment 2a, † 25°	Experiment 2b, † 37°	Experiment 2c, 37°
0	121	120	120			
6	121	122	123	27	68	49
12	126	126	124	57	121	72
24	120	122	113	93	232	
30	122	115	103*	123	278	*
34½		114	99.0		303	17
38		114	91.7		319	23
pH‡	7.50	6.92	7.51			

\* Glucose added.

† Glucose added at the beginning of the experiment.

‡ pH values taken at the end of the experiment.

(The drop in the potassium content at 30 hours in Experiment 2b is unaccounted for, inasmuch as that level was maintained for the next 8 hours. In the experimental procedure this blood was apportioned into two flasks, the sampling of the second flask being begun at 30 hours.) In Experiments 2a, 2b, and 2c a rise in the potassium content of the cells was observed during the early

periods, which is not to be attributed to dehydration.<sup>3</sup> Such an observation was also made by Danowski (22).

The potassium content of fresh human erythrocytes does not begin to fall rapidly as soon as the glucose has disappeared. This is seen in Fig. 6. Here the glucose was removed by washing the cells of defibrinated blood twice with 0.154 M sodium chloride buffered with M/150 phosphate to pH 7.3. The cells were then diluted to approximately the original volume with this solution

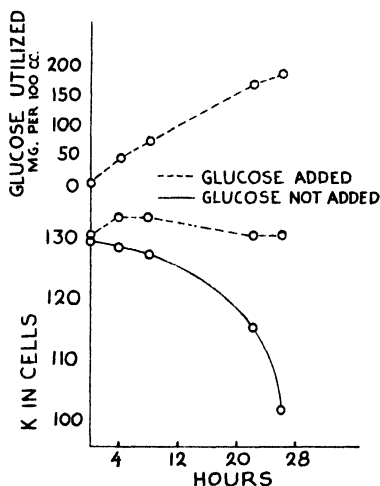


FIG. 6. The effect of glucose on the potassium content of washed human erythrocytes suspended in buffered saline. Ordinate, mg. of potassium in the cells of 100 ml. of mixture.  $T = 37^\circ$ . The sample to which glucose was added showed 0.36 per cent hemolysis after 26 hours incubation, the mixture without glucose being 2.1 per cent hemolyzed at that time.

and the sample divided, glucose being added to one portion, and placed at  $37^\circ$ . The cells suspended in a medium without glucose maintained their potassium content (the loss is less than 2 per cent) for at least 8 hours. Thereafter, the loss was greater, the

<sup>3</sup> To allow a free diffusion of  $\text{CO}_2$  and thus a more stable pH the flasks were stoppered with cotton plugs. Thus a certain amount of dehydration was possible at  $37^\circ$  when a covered water bath was not used. This dehydration was found in a few control experiments to be of such a magnitude as to account for a concentrating of from 0 to 4 per cent during 24 hours. As each experiment has its own control, however, dehydration may be ignored.



rate increasing in an autocatalytic fashion. The cells washed free of serum not only maintained their ability to utilize added glucose but also showed no loss of potassium for at least 24 hours.

However, this pattern does not occur throughout, for the cells of a citrate-glucose-blood mixture showed a relatively small but rapid loss of potassium during the first few hours after mixing, the extent being approximately the same at both 25° and 37°. Thus, at 25° the cells of 100 ml. of this mixture contained 54.7, 47.9, and 46.0 mg. of potassium after 0, 5½, and 11 hours respectively; the

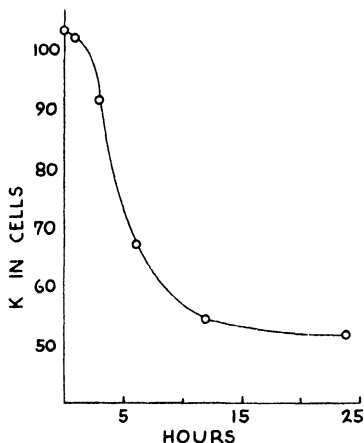


FIG. 7. The effect of 0.042 M sodium fluoride on the potassium content of the cells of a fresh citrate-blood mixture. Ordinate, mg. of potassium in the cells of 100 ml. of mixture.  $T = 25^\circ$ .

corresponding values at 37° were 54.7, 46.0, and 44.1 mg. This loss is probably analogous to that shown by Maizels (9) and Davson (10) in non-electrolyte solutions, although the influence of swelling (23) cannot be ignored. Whatever may be its explanation, it appears to bear no relationship to the other effects described, and exemplifies the fact that alteration of the cation content of erythrocytes, such as has been frequently observed under varying conditions, is not necessarily to be attributed to a depletion of glucose or an altered metabolic function. After storage for 7 days the cells of a citrate-glucose-blood mixture showed little tendency to increase in potassium content when placed at 37°; no loss was observed for 24 hours.

*Effect of Sodium Fluoride*—Wilbrandt (24, 25) showed that the osmotic resistance of human erythrocytes increased markedly after a short incubation at 37° in saline solution containing fluoride or iodoacetate. He interpreted this as being due to loss of potassium, a fact he verified by actual analysis. Davson and Danielli (26) were unable to find a loss from rabbit erythrocytes (the only mammalian species studied) after suspension in fluoride solution for 1 hour at 25°, although red blood cells of the goose showed a slight depletion of potassium under the same conditions.

Observations in this laboratory have shown that fluoride (0.02 to 0.05 M) causes a marked loss of potassium from the erythrocytes of both fresh and stored human blood. One such observation is shown in Fig. 7. The relatively small change during the 1st hour may explain the failure of Davson and Danielli to observe a fluoride effect.

#### DISCUSSION

Plainly, the view that the erythrocyte membrane is impermeable to cations, the distribution of these ions being a static phenomenon, must be abandoned. In its place must be substituted a concept of normal membrane permeability to sodium and potassium, their concentrations in the cells being maintained by one or more metabolic functions of the cells. Thus, when the metabolic level is lowered, as by chilling, potassium and sodium will diffuse with the concentration gradient. Partial restoration of that activity by raising the temperature will lead to a redistribution of the cations toward their normal equilibrium. The maintenance of a more or less normal metabolic level by the addition of glucose keeps the cation distribution at existing levels when fresh blood is kept at room or body temperatures. Depletion of glucose sooner or later apparently leads to a loss of the ability of the red cells to concentrate these cations. This concept would imply that there is normally an exchange between the cations inside and outside the cells, a hypothesis which finds confirmation in the experiments with radioactive ions mentioned previously.

It seems likely that glycolysis, *per se*, is not the metabolic function responsible for the migration of potassium against a concentration gradient. Thus, fresh erythrocytes appear to maintain their potassium content as well at 25° as at 37° so long as glucose is present, although the glycolytic rate at the lower

temperature is only half as great. Moreover, the depletion of glucose is not followed by an immediate loss of potassium. On the other hand, the accumulation of this ion by erythrocytes which have been stored at refrigerator temperatures is greater if the cell is actively glycolyzing. It would seem that the activity concerned in the accumulation of potassium within the erythrocytes must be enhanced during glycolysis and persist for a while after glucose has been depleted. There is little indication of what this activity might be or how it functions. Diphosphoglycerate may be involved, since it is said to undergo formation and decomposition during red blood cell glycolysis (27) and to persist after the disappearance of glucose. The metabolic function controlling sodium distribution is not necessarily the same as that controlling potassium.

Wilbrandt (25) showed that, whereas moderate concentrations of fluoride (0.02 to 0.05 M) exerted a maximal effect on the loss of potassium, higher concentrations (M/7) tended to inhibit this loss. Davson (personal communication) has made the same observation. It would seem, therefore, that the potassium loss which fluoride induces is not to be attributed to an inhibition of the metabolic function normally responsible for the maintenance of cation distribution. Wilbrandt suggested that the loss of potassium seen with fluoride was due to an induced cation permeability in the red blood cell membrane. This permeability resulted from a side reaction of glycolysis which was itself inhibited in higher concentrations of fluoride. However, such a concept would imply that the membrane is normally impermeable to potassium, a view which is not in harmony with the present work. (The possibility of a simultaneous inhibition of the activity controlling the cation equilibrium and alteration of permeability cannot be denied.) Moreover, Wilbrandt showed that the effect of fluoride in moderate concentrations was quickly overcome when pyruvate was added (at least the potassium loss was stopped). Thus, at present, it seems logical to assume that the loss of potassium which fluoride induces results from an inhibition of the metabolic function normally responsible for the potassium accumulation. The explanation of the effect of high concentrations of fluoride then must await further experimentation. In this connection it should be remembered that fluoride has a certain corrosive action on animal membranes (28).

It is believed that a cation impermeability of the erythrocytes prevents their rupture by the osmotic force of the Gibbs-Donnan equilibrium. However, the same result can be achieved, although in a different way, if the metabolic activity of the cells controls the distribution of cations, even though the membrane be considered permeable to sodium and potassium. Indeed, because of this activity and the apparently very slow rate of permeation of cations, as compared to anions, the membrane may be said to be functionally impermeable to the positive ions, at least as regards such functions as the transport of  $\text{CO}_2$ . Thus, the calculations of Van Slyke *et al.* (29) and more recently of Rapoport and Guest (30), showing that the anions of blood tend to distribute themselves according to the Donnan equilibrium (assuming cation impermeability), lose none of their significance. Moreover, inhibition of the functions controlling the erythrocyte cation content should lead to such a swelling. This has been observed to occur following fluoride poisoning (25) and also during storage at refrigerator temperatures, as will be shown in a forthcoming paper.

#### SUMMARY

Some of the potassium which is lost from human erythrocytes when a citrate-blood mixture is stored at refrigerator temperatures reenters the cell when the mixture is placed at  $25^\circ$  or  $37^\circ$ . The addition of glucose increases the amount of potassium which will reenter if the glucose is utilized.

When a stored citrate-blood mixture is placed at  $37^\circ$ , sodium decreases as potassium increases within the cell so long as glucose is being utilized. If no glucose is present, sodium enters the cell, moving with the potassium, for a period of 12 hours. At 24 hours the potassium content has again fallen.

The cells of blood to which glucose has been added maintain their potassium content much longer at  $37^\circ$  than when the glucose has been depleted, although the potassium loss is not appreciable for some hours after glucose has been removed by washing.

Sodium fluoride, 0.02 to 0.05 M, causes a rapid loss of potassium from the erythrocytes of both fresh and preserved bloods at  $25^\circ$  and  $37^\circ$ .

It is concluded that the normal distribution of cations between the human erythrocyte and the extracellular medium is main-

tained by some metabolic activity of the cell. So far as the potassium is concerned, the fundamental factor does not appear to be glycolysis *per se*. The metabolic activities controlling the sodium and potassium distribution are not necessarily the same. The action of fluoride is tentatively concluded to be inhibition of the responsible metabolic function.

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# AUTOXIDATION OF STEROLS IN COLLOIDAL AQUEOUS SOLUTION

## THE NATURE OF THE PRODUCTS FORMED FROM CHOLESTEROL

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The study reported in this paper was originally undertaken as a sequel to the isolation from pregnant mare's serum of 7( $\beta$ )-hydroxycholesterol (1), a sterol which had previously not been encountered in nature, although its epimer, 7( $\alpha$ )-hydroxycholesterol, had been shown by Haslewood (2) to occur in ox liver, and by MacPhillamy (3) in hog liver. More recently we have found the latter isomer also in pregnant mare's serum.<sup>1</sup> Both these compounds give a positive Lifschütz reaction, and to this extent may be considered as identical with the ill defined "oxycholesterol" which Lifschütz (4) claimed to be present as a preformed entity in the unsaponifiable matter of blood and tissues. Lifschütz apparently took it for granted that this preformed "oxycholesterol" and the chromogen which he obtained from cholesterol by various, mostly oxidative, procedures *in vitro* were represented by one and the same substance. This notion has now been dispelled not only by the isolation of two pure chromogens from natural sources, but also by the preparation in recent years of numerous other cholesterol derivatives of known constitution, which all give the Lifschütz reaction. However, besides these well defined compounds there exists a type of "oxycholesterol," the chemical nature of which has never been elucidated; namely, the chromogen formed from

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<sup>1</sup> Wintersteiner, O., and Ritzmann, J. R., unpublished data.



cholesterol by the combined action of heat and oxygen. This was of concern to us in so far as we could not be absolutely certain that, in spite of the precautions taken, some accidental autoxidation of cholesterol had not occurred during the fractionation of the serum and given rise to one or the other of the two chromogenic diols isolated. An inquiry into the nature of the chromogen formed by autoxidation particularly under conditions such as might prevail at one time or other during the isolation process seemed in order.

Of special interest to us in this connection was the work of Blix and Löwenhielm (5) on account of the comparative mildness of the conditions employed and the high yields of "oxycholesterol" reported. These authors aerated colloidal aqueous solutions of cholesterol under various conditions and determined the amount of chromogen formed by quantitative application of the Lifschütz reaction. We adapted their colorimetric procedure to our purposes, with pure 7( $\beta$ )-hydroxycholesterol as standard,<sup>2</sup> and repeated some of their experiments on a preparative scale, using cholesterol sols stabilized with sodium stearate. At 85°, which was chosen as the standard temperature for subsequent work, the amount of chromogen formed after 2 hours was consistently 25 to 30 per cent of the cholesterol present, and could not be raised by further aeration. In working up the reaction mixtures only about 20 per cent of still highly impure cholesterol could be recovered in crystalline form. The mother liquor material was acetylated and subjected to chromatographic separation. The fractions with the highest chromogen content were hydrolyzed and benzoylated, and yielded 7( $\alpha$ )-hydroxycholesterol dibenzoate without any difficulty.

In the search for other autoxidation products we were aided by ultraviolet absorption measurements, which revealed the presence of two distinct light-absorbing entities. The extinction was particularly high in the region around 240 m $\mu$ , characteristic for  $\alpha$ ,  $\beta$ -unsaturated ketones, while the other band, with a maximum of 280 m $\mu$ , was much less intense. Fractionation with Girard's

<sup>2</sup> The details of the colorimetric procedure will be given in a subsequent paper. The "color equivalent" referred to in the text and in Table I gives the chromogenic potency of the unknown sample as per cent of that of 7( $\beta$ )-hydroxycholesterol.

reagent showed that large amounts of ketones were indeed present. 75 per cent of the ketonic fraction was found to be digitonin-precipitable; decomposition of the digitonide yielded almost pure 7-ketocholesterol. This compound thus accounted for most, if not all, of the light absorption around 240 m $\mu$ .

These findings, already communicated in a preliminary note (6), have now been extended to include the isolation of two additional compounds. In the fractionation of larger amounts of the autoxidized mixture the ketones were removed as far as possible with Girard's reagent. The "non-ketonic" fraction, which contained all of the chromogenic material, was acetylated and subjected to chromatographic analysis. This effected first of all complete removal of the remaining cholesterol; furthermore it showed that besides 7( $\alpha$ )-hydroxycholesterol, which is dextro-rotatory, a strongly levorotatory chromogen was present. The trend of the rotation values in the eluted chromogenic fractions (*cf.* Table I) almost exactly paralleled that in experiments which we had previously carried out with similar material from pregnant mare's serum. The highly levorotatory fractions in the latter series had given good yields of crystalline 7( $\beta$ )-hydroxycholesterol directly on hydrolysis by alkali. The corresponding fractions from the autoxidation experiments, however, proved to be much less tractable. Further fractionation of the levorotatory chromogens by repetition of the chromatographic procedure proved necessary to obtain crystalline products. In one run this step yielded fractions of exceptionally high levorotation ( $-190^\circ$  to  $-210^\circ$ ), which crystallized partly on standing. However, after purification the crystalline product failed to give the Lifschütz reaction; it was identified as 7-keto- $\Delta^{3,5}$ -cholestadiene (7-keto-cholesterilene). This compound is undoubtedly responsible for the major part of the light absorption at 280 m $\mu$ . Its unexpected occurrence in the "non-ketonic" fraction is to be attributed to the incompleteness of separation in the Girard process.

In subsequent runs, in which more attention was paid to this difficulty, we were able to secure levorotatory chromogenic fractions with a reasonably low content of light-absorbing entities. Eventually a small amount of a crystalline chromogen melting at 137°<sup>3</sup> was obtained. Since this product had the approximate

<sup>3</sup> All melting points given in this paper are corrected.

composition of a cholestenediol and showed the correct Lifschütz equivalent (98 per cent), it was reasonable to assume that we were dealing with 7( $\beta$ )-hydroxycholesterol, contaminated with some of the  $\alpha$  epimer (7). Later work showed that this conclusion was premature. The substance in question, here provisionally named Compound A, is a new isomer melting at 139–140°, which is not identical with any of the known cholestenediols. When pure it crystallized from methanol in characteristic hexagonal plates, quite distinct from the needle-shaped crystals of 7( $\beta$ )-hydroxycholesterol. Other points of difference are its much higher levorotation ( $-134^\circ$ ) and the lack of precipitability by digitonin. Furthermore only one hydroxyl group was demonstrable by esterification; the esters prepared were the monobenzoate (m.p.  $117^\circ$ ) and the mono-3,5-dinitrobenzoate (m.p.  $164^\circ$ ). The compound absorbs bromine equivalent to one double bond. It is not attacked by lead tetraacetate.

The properties of the new substance suggested that it may have arisen from 7( $\beta$ )-hydroxycholesterol by rearrangement either during the primary reaction or during one of the steps in the isolation procedure. As to the first possibility, we convinced ourselves that 7( $\beta$ )-hydroxycholesterol is not changed under the conditions of the autoxidation reaction. Treatment with Girard's reagent was tried next. All of the material was recovered in the non-ketonic fraction, which on crystallization from methanol yielded Compound A in almost pure form. The same result was obtained by boiling a solution of the  $\beta$ -diol in alcohol containing 10 per cent acetic acid for 2 hours. 7( $\alpha$ )-Hydroxycholesterol, on the other hand, is not at all affected by this treatment. These results leave hardly any doubt that 7( $\beta$ )-hydroxycholesterol is one of the products formed in the autoxidation reaction, and that it was transformed into Compound A during the Girard separation. In our work on pregnant mare's serum the 7( $\beta$ ) isomer could be isolated as such because the fractions containing the chromogenic diols were obtained by a procedure which did not include treatment with Girard's reagent.

The fact that the rearranged product is recovered in the non-ketonic fraction proves that Compound A contains no, or no active, keto group. This was confirmed by treatment, under rigorous conditions, with semicarbazide acetate; the compound was recovered unchanged.

The facts so far ascertained favor the formulation of Compound A as a cholestenediol in which one hydroxyl group is situated at a tertiary carbon atom, probably C<sub>6</sub>. Allocation to this position would also furnish an explanation for the loss of precipitability by digitonin. While an additional hydroxyl group in positions 4, 6, or 7 does not interfere with the digitonin reaction, such a group when situated at C<sub>6</sub> apparently exerts a hindering effect, since  $\beta$ -cholestanetriol-3,5,6 does not yield an insoluble digitonide (8). The same difference is evident in the behavior of 6-methylcholesterol, which reacts with digitonin, and of 6-methylcholestanediol-3,5, which fails to do so (9). We therefore tentatively assume that Compound A is a  $\Delta^6$ -cholestenediol-3,5 formed from the 3,7-diol by a rearrangement of the allylic type. Work to substantiate the proposed structure is in progress.

#### EXPERIMENTAL

*Preparation and Aeration of Colloidal Cholesterol Solutions*—The cholesterol used was recrystallized from methanol till the Lifschütz reaction was negative. It contained no spectrographically demonstrable amounts of 7-ketocholesterol. Our first experiments (7) were carried out on 0.05 per cent cholesterol sols, but we soon found that qualitatively and quantitatively identical results could be obtained with colloidal solutions containing up to 0.5 per cent cholesterol. The sols were prepared directly in the reaction vessel, a round bottom three neck flask holding 3 liters and fitted with an efficient mechanical stirrer. One of the side tubules accommodated the air inlet tube ending in a bulb with fine holes, while the other held a thermometer. The flask rested on a steam bath permitting the maintenance of the reaction temperature (85°) by adjustment of the steam intake. 1 gm. of sodium stearate was dissolved in 1 liter of water previously warmed to 70–80° and the pH adjusted to about 8.5 with dilute hydrochloric acid. A hot solution of 5 gm. of cholesterol in 200 cc. of absolute ethanol was slowly added to the vigorously stirred contents of the flask. The stirring was continued for 5 hours at 85°  $\pm$  2° while a slow stream of air passed through the solution. The alcohol condensing in the upper part of the flask effectively prevented the escape of foam through the central mouth.

The results obtained by this simple procedure are well reproducible. In a subsequent paper we shall report a series of

experiments in which we tested the influence of various experimental factors on the rate and extent of formation of the principal reaction products, 7-ketocholesterol and the chromogens. These studies revealed that the yields of these entities are not appreciably affected by variation, within rather wide limits, of oxygen pressure, speed of aeration, pH, substrate concentration, nature and purity of the detergents used, and the mode of preparation of the sol. This explains the absence of a more rigorous control of these variables in the procedure described above.

*Fractionation of Reaction Products*—The aerated solution was cooled, transferred to a separatory funnel, acidified with hydrochloric acid, and extracted with 500 cc. of ether. The ether phase was washed three times with about 100 cc. of a 10 per cent potassium hydroxide solution, and then with several portions of water till the ether became clear. The residue from the dried ether solution (4.8 to 5.2 gm.) was treated with Girard's Reagent T (5 gm.) in the customary manner and separated into ketonic and non-ketonic fractions.

The ketonic fraction generally accounted for 25 to 30 per cent of the weight of the starting material. Small additional amounts of ketones (about 5 per cent) could be recovered by subjecting the non-ketonic fraction to a second treatment with the reagent. The ketonic material was always completely free of chromogenic compounds.

*7-Ketocholesterol*—The partly crystalline ketonic fraction (1.4 gm.) was purified by dissolving it in a small amount of ether and adding several volumes of pentane. The crystalline precipitate was filtered and washed with cold pentane (840 mg., m.p. 165–168°). Two recrystallizations from the same solvents yielded needles which melted at 170–172°. The mother liquor material was acetylated, which permitted the recovery of additional amounts of the ketone in the form of the less soluble acetate.

*Analysis*— $C_{27}H_{44}O_2$ . Calculated. C 80.93, H 11.08

Found. " 80.63, " 10.83

$[\alpha]_D^{25} = -104^\circ$  (in chloroform)

Free 7-ketocholesterol has been previously described by Mauthner and Suida (10), who give its melting point as 157°, and by Ogata and Kawakami (11), who report 163–164°.

We saponified the acetate, prepared according to Windaus, Lettré, and Schenk (12), with potassium carbonate in 80 per cent methanol at room temperature in order to avoid formation of 7-keto- $\Delta^{3,5}$ -cholestadiene which might occur in the hydrolysis with hot caustic alkali. The free ketosterol thus obtained showed the same melting point and specific rotation as the product isolated from the autoxidized mixture. The melting point of the mixed sample was not depressed.

The absorption spectrum of the compound exhibits the band typical for  $\alpha, \beta$ -unsaturated ketones (in ethanol  $\epsilon$  at  $\lambda$  238  $m\mu$  = 12,500).

The acetate prepared from the isolated ketone melted at 157–159°, as did the reference preparation.

*Analysis*— $C_{29}H_{46}O_3$ . Calculated. C 78.76, H 10.48  
Found. “ 78.55, “ 10.47  
 $[\alpha]_D^{25} = -97^\circ$  (in chloroform)

When the crude ketonic fraction from another run was treated with digitonin in 90 per cent alcohol, about 75 per cent of its weight was precipitated. The product regenerated from the crystalline digitonide consisted of pure 7-ketocholesterol. The material recovered from the digitonide filtrate was a non-crystallizable oil. Its absorption spectrum indicated that it contained 7-ketocholestadiene, but also some 7-ketocholesterol which had escaped precipitation. Since the latter compound preponderated in the mixture, the isolation of the diene from this fraction was not attempted.

*Non-Ketonic Fraction*—This fraction contained all of the chromogenic material (about 40 per cent of its weight), the unattacked cholesterol (about 50 per cent), and ketones which had escaped the Girard reaction (5 to 10 per cent). The mixture was acetylated with acetic anhydride and pyridine at room temperature. The acetylated product was dissolved in pentane, adsorbed on a column of Brockmann's aluminum oxide, and fractionally eluted with mixtures of pentane and benzene, benzene, and finally acetone, as exemplified in Table I.

*7-Keto- $\Delta^{3,5}$ -Cholestadiene*—In the experiment recorded in Table I the amount of residual ketones was exceptionally low, since the autoxidation products had been treated twice with Girard's re-

gent. In other runs, which had received but one such treatment, 7-ketocholesterol was spectrographically demonstrable in all the

TABLE I

*Example of Chromatographic Separation of Non-Ketonic Material (0.98 Gm.)*

Column  $250 \times 18$  mm., Brockmann's aluminum oxide, holding about 40 cc. of solvent. The eluates were cut for each 40 cc. of ingoing solvent.

Fraction No.	Solvents (ingoing)	Weight of fraction	$[\alpha]_D$ (chloro- form)	Color equiva- lent (cf. foot- note 2)	Compounds isolated
		mg.	degrees		
1	Substance in pentane	0			
2	" " "	0			
3	" " "	1			
4	Benzene-pentane, 1:9	37			
5	" " " 1:9	101	-45	0	Crystalline cholesterol acetate
6	" " " 1:9	108			
7	" " " 1:9	54			
8	" " " 2:8	36			
9	" " " 2:8	28	-69	0	Compound A
10	" " " 2:8	16			
11	" " " 2:8	29	-82	84	
12	" " " 3:7	32	-91	115	
13	" " " 3:7	34	-107	150	
14	" " " 4:6	33	-62		
15	" " " 4:6	44	-77	100	
16	" " " 4:6	17			
17	" " " 4:6	9			7( $\alpha$ )-Hydroxy- cholesterol
18	" " " 6:4	7			
19	" " " 6:4	22	-70	54	
20	" " " 6:4	19			
21	" " " 6:4	18			
22	Benzene	17			
23	" " "	48	+20	93	
24	" " "	27			
25	" " "	22			
26	Acetone	83	-3	50	
27	" " "	115	+2	42	
28	" " "	2			

eluates past those containing the cholesterol, the major part appearing in the acetone washings. This was true also of the entity absorbing around  $280\text{ m}\mu$ , but in some cases the levorotatory

chromogenic fractions washed out by pentane-benzene 8:2 and 6:4 also exhibited fairly intense absorption in this region. It was material of this description from which we isolated, more or less accidentally, the substance responsible for this absorption, 7-keto- $\Delta^{3,5}$ -cholestadiene.

1.4 gm. of such a product were readsorbed on aluminum oxide and eluted in the usual manner. Three of the fractions eluted with pentane-benzene 8:2 showed an unusually high levorotation ( $-195^\circ$  to  $-210^\circ$ ), and on standing in the desiccator crystallized in part. The crystals were freed from oily by-products by washing with a little cold methanol. Two recrystallizations from the same solvent yielded plates melting at  $109-111^\circ$ . The melting point remained unchanged on admixture of an authentic sample of the ketodiene which had been prepared from 3-chloro-7-keto- $\Delta^5$ -cholestene according to Marker and coworkers (13).

*Analysis*— $C_{27}H_{42}O$ . Calculated. C 84.75, H 11.07

Found. " 84.38, " 10.90

$[\alpha]_D^{25} = -266^\circ$ ; reference preparation,  $-279^\circ$

$\epsilon$  at  $\lambda$  280  $m\mu$  = 23,000 (in ethanol)

*7( $\alpha$ )-Hydroxycholesterol Dibenzoate*—Fractions 23 to 25 (Table I), together 97 mg., were hydrolyzed with 4 cc. of 5 per cent methanolic potassium hydroxide at room temperature for 2 days. The hydrolysis product (79 mg.) was treated with 1.5 cc. of pyridine and 0.5 cc. of benzoyl chloride. After standing overnight at room temperature the mixture was worked up in the usual manner. The benzoylated product crystallized on short boiling with methanol, yielding 70 mg. of needles melting at  $164-169^\circ$ . Recrystallization from the same solvent raised the melting point to  $171-172.5^\circ$ .

The preparation obtained in our preliminary experiment melted at  $174^\circ$ . It did not depress the melting point ( $174^\circ$ ) of an authentic specimen prepared according to Windaus, Lettré, and Schenk (12).

*Analysis*— $C_{41}H_{64}O_4$ . Calculated. C 80.60, H 8.92

Found. " 80.78, " 9.02

$[\alpha]_D^{25} = +94.3^\circ$  (in chloroform); reference preparation,  $+92.8^\circ$

*Compound A*—Fractions 11, 12, and 13 (Table I) were combined and hydrolyzed in the usual manner with cold methanolic potas-



sium hydroxide solution. On standing, the dark brown solution deposited crystals, which were separated by filtration and washed with methanol. The product (82 mg.) melted unsharply at  $140^{\circ}$ . Its purification proved difficult and wasteful. The final recrystallization from methanol-ether left only 7 mg. of needles (m.p.  $137^{\circ}$ ) possessing the approximate composition of a cholestenediol and giving a color equivalent of 98 per cent in the Lifschütz reaction. It is now clear that this substance was not, as we then assumed (7), 7( $\beta$ )-hydroxycholesterol contaminated with some of the  $\alpha$  epimer, but Compound A.

In an attempt to secure larger amounts of the supposed  $\beta$ -diol 11 gm. of "non-ketones" were worked up in the manner described. The levorotatory chromogens (1.1 gm.,  $[\alpha]_D = -80^{\circ}$  to  $-100^{\circ}$ ) were rechromatographed, but no further appreciable fractionation was accomplished in this step, as the rotation and chromogen values of most of the eluates did not deviate markedly from those of the starting product. The fractions eluted with pentane-benzene 8:2 (264 mg.), when hydrolyzed in the usual manner at room temperature and then allowed to stand in the refrigerator, yielded a copious deposit of rosettes of needle-shaped crystals (215 mg., m.p.  $135^{\circ}$ ). On recrystallization from methanol large hexagonal plates melting at  $139$ – $140^{\circ}$  were obtained. Further recrystallization did not change the melting point.

<i>Analysis</i> — $C_{27}H_{46}O_2$ .	Calculated.	C 80.52, H 11.52
	Found.	" 80.84, " 12.07
		" 80.52, " 11.62

A sample of this preparation was sublimed in a high vacuum at  $120$ – $130^{\circ}$ . The crystalline sublimate, which melted at  $137^{\circ}$ , was analyzed without further purification; found, C 81.19, H 11.77 and C 81.09, H 11.81;  $[\alpha]_D^{24} = -134^{\circ}$  (in chloroform); Lifschütz color equivalent about 150. The compound is transparent in the ultraviolet region above  $220\text{ m}\mu$ .

The compound did not precipitate with digitonin in 90 per cent ethanol, and showed the original melting point after recovery from the mixture by ether extraction.

The determination of the bromine uptake was carried out according to Ralls (14); 6.99 mg. (0.0174 mm) within 30 minutes at  $0^{\circ}$  consumed 0.0366 milliequivalent of bromine and liberated

0.0033 milliequivalent of acid; calculated for one double bond 0.0348 milliequivalent of Br.

*Monobenzoate*—Benzoylation with benzoyl chloride and pyridine at room temperature yielded an inhomogeneous product. 29 mg. were therefore treated with 0.2 and 1.5 cc. respectively of these reagents for 3 hours at 80°. The reaction product was recovered by ether extraction, leached with cold methanol, and then recrystallized from ethanol. 26 mg. of needles melting at 109–110° were obtained. After two more recrystallizations from methanol the melting point became constant at 116–117°.

$C_{34}H_{50}O_3$ .	Calculated.	C 80.58, H 9.95
	Found.	" 80.72, " 10.09
		" 80.69, " 10.13

*Mono-3,5-Dinitrobenzoate*—25 mg. were treated with 100 mg. of the acid chloride and pyridine as described for the benzoate. The reaction product (25 mg.) was recrystallized from methanol-benzene to yield small needles melting at 163–164°. Further recrystallization did not change the melting point.

<i>Analysis</i> — $C_{34}H_{48}O_7N_2$ .	Calculated.	C 68.44, H 8.11, N 4.69
	Found.	" 69.40, " 8.33, " 4.82

The high carbon value was not improved in the analysis of another preparation of the same melting point, which had been subjected to additional purification by the chromatographic method.

Found.	C 69.30, H 8.58, N 4.72
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*Rearrangement of 7(β)-Hydroxycholesterol to Compound A*—A solution of 50 mg. of the diol in 5 cc. of absolute alcohol containing 10 per cent acetic acid and 50 mg. of Girard's Reagent T was boiled under a reflux for 2 hours. The mixture was poured into ice water containing sodium hydroxide equivalent to nine-tenths of the acetic acid, and was extracted with ether. The ether residue, a colorless oil weighing 50 mg., crystallized on addition of methanol. The crystals were collected and washed with cold methanol (25 mg., m.p. 136–137°). After recrystallization the melting point was 137–138°. A mixture with Compound A melted at the same temperature.

50 mg. of 7(β)-hydroxycholesterol were treated in the same

manner, except that Girard's reagent was omitted; 25 mg. of Compound A melting at 136° were obtained.

Benzoylation of 38 mg. of the rearrangement product yielded 42 mg. of the monobenzoate melting at 115–116°.

#### DISCUSSION

While it has been known for some time that cholesterol in colloidal aqueous solution is very susceptible to the action of molecular oxygen (5, 15, 16), our experiments have now for the first time thrown light on the nature of the resulting autoxidation products. The results may be summarized by stating that the main point of attack is position 7 in the cholesterol molecule, and that the conversion into 7-substituted compounds takes place with surprising ease and to a much greater extent than could be foreseen. Theoretical considerations regarding the probable chemical mechanism of the autoxidation reaction will be presented in a subsequent publication. Here we wish to discuss more specifically the biological implications of our findings. In this connection it should be mentioned that the formation of 7-ketocholesterol and of the chromogens proceeds just as well at 37° as at 85°, although at a much slower rate (7). In fact the conversion seems to be more complete at the lower temperature, which is due to an increased production of the ketone. There is no reason, then, why this type of autoxidation should not also occur *in vivo*, as its prerequisites, cholesterol held in colloidal solution and high oxygen pressures, certainly obtain in biological systems. Furthermore, it must be remembered that 7-ketocholesterol and 7( $\alpha$ )-hydroxycholesterol are intermediates in the laboratory preparation of 7-dehydrocholesterol, provitamin D<sub>3</sub> (12), which has been shown by Windaus and collaborators to occur in various animal tissues (17), and has been isolated in crystalline form from pig skin (18). The formation of provitamin D-containing products from cholesterol by autoxidative measures has been often described; it is reasonable to assume that the two compounds mentioned are concerned as intermediates in this process, and that this may also be the case in the biological formation of the provitamin. This premise granted, our present results do not necessarily prove that the compounds isolated from mare's serum, to which we have recently added 7-ketocholesterol,<sup>1</sup> must have

arisen *in toto* by autoxidation outside of the body. On the other hand, it is highly probable that at least *a part* of the isolated products were formed *in vitro* during the initial phases of the isolation procedure, when soaps were still present. Our recent observation that the addition of cyanide prevents the autoxidation reaction may be helpful in deciding the question whether, or to what extent, these compounds occur preformed in the biological starting materials.

#### SUMMARY

Cholesterol when aerated at 85° in colloidal aqueous solution is for the major part transformed into a mixture of compounds, of which 7-ketocholesterol, 7-keto- $\Delta^{3,5}$ -cholestadiene, and 7( $\alpha$ )-hydroxycholesterol have so far been identified. The chief reaction product is 7-ketocholesterol, which accounts, on the basis of spectrographic data, for about 40 per cent of the cholesterol used, and has been actually recovered in yields of 20 to 25 per cent. A fourth compound (m.p. 139–140°) isolated from the reaction mixture was shown to be a rearrangement product of 7( $\beta$ )-hydroxycholesterol formed from the latter on treatment with Girard's reagent during the fractionation procedure. The new substance is not identical with any of the known cholestenediols. Its properties and probable structure are discussed.

These results show that position 7 in cholesterol is extremely susceptible to attack by molecular oxygen, while the 3-hydroxyl group is not involved in this type of oxidation.

Certain biological implications of these findings, especially their bearing on the recent isolation of 7-substituted derivatives of cholesterol from blood and tissues, are discussed.

The analyses of Compound A and derivatives were carried out by Mr. J. F. Alicino, Fordham University, the rest by Mr. William Saschek, Columbia University.

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## THE COENZYME I CONTENT OF RAT TISSUES IN EXPERIMENTAL HYPERTHYROIDISM\*

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The marked stimulatory effect of the secretion of the thyroid gland upon the basal metabolic rate is generally recognized. A fuller understanding of this general phenomenon has been sought through *in vitro* studies on the metabolism of surviving tissues from hyperthyroid animals. Thus, a number of workers (1-3) have observed marked increases in the oxygen consumption of tissues from animals in which a state of hyperthyroidism had been experimentally induced. Also, evidence has been presented to show that the addition of thyroxine to tissue slices may result in an increased oxygen consumption (4, 5). This relationship of the thyroid hormone to respiratory processes has prompted studies on the effect of the hormone upon the individual components of such respiratory systems. Cohen and Gerard (1) have observed appreciable increases in the dehydrogenase activities of various systems studied in tissues from hyperthyroid rats. Klein (6) reported an increase in the *d*-amino acid oxidase content of liver from hyperthyroid rats and showed later (7) that the increase in activity was probably due to an increase in the protein component of the oxidase. Peters and Rossiter (8) noted that hyperthyroidism caused a fall in the cocarboxylase content of rat tissues. This effect could be overcome by the administration of increased doses of thiamine.

As an extension of the general subject of the relationship of hyperthyroidism to respiratory catalysts, the present investigation

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was undertaken to study the effect of experimentally produced hyperthyroidism upon the coenzyme I content of rat tissues.

A great deal of evidence has been accumulated to show that the requirements for vitamin A (9-11), ascorbic acid (12, 13), thiamine (14, 15), and possibly other members of the vitamin B complex (14, 15) are increased greatly in a state of hyperthyroidism. In this connection it became of interest to investigate the requirement of the rat for nicotinic acid in hyperthyroidism and also to study

TABLE I  
*Composition of Rations*

Components	Ration I*	Ration II†	Ration III‡
Yellow corn, <i>gm.</i> .....	71	71	
Labco casein, ".....	18	18	18
Sucrose, <i>gm.</i> .....			76
Salts (16), <i>gm.</i> .....	4	4	4
Cottonseed oil, <i>gm.</i> .....	5	5	
Cod liver " ".....	2	2	
Corn oil, <i>gm.</i> .....			2
Thiamine, ‡ <i>mg.</i> .....	0.30	2	2
Pantothenic acid, <i>mg.</i> .....		2	2
Riboflavin, <i>mg.</i> .....	0.30	1	1
Pyridoxine, ".....	0.30	1	1
Choline, <i>mg.</i> .....			100

\* 1 drop of haliver oil was fed weekly to each rat.

† 4 drops of haliver oil were fed weekly to each rat.

‡ We are indebted to Merck and Company, Inc., for generous supplies of thiamine, pantothenic acid, riboflavin, pyridoxine, nicotinic acid, and choline.

the effect of excessive dosages of nicotinic acid in counteracting any deleterious action of the hyperthyroid condition upon the coenzyme I content.

### *Procedure*

Albino rats taken from the stock colony and weighing from 200 to 250 gm. were employed in Experiments A and B, while weanling rats were employed in Experiment C. The rats were housed in individual wire-bottomed cages and were weighed weekly. The composition of the rations which were fed *ad libitum* is given in Table I.

Desiccated thyroid was fed at a level of 200 mg. per day for periods of time varying from 3 to 4 weeks. The daily dosage of dried gland was mixed with a small amount of the ration and food was withheld from the rat until this portion was consumed. This procedure insured complete consumption of the desiccated thyroid. The thyroxine solution, prepared by dissolving thyroxine<sup>1</sup> in the minimum amount of 0.1 N NaOH and diluting to volume with distilled water, was administered daily by subcutaneous injection over varying periods of time at levels ranging from 0.20 to 20 mg. per kilo of body weight.

Determinations of the coenzyme I content of liver, kidney cortex, and in some instances of thigh muscle were made by the yeast fermentation method described by Axelrod and Elvehjem (17). Recovery experiments which were carried out on a series of tissue extracts from the hyperthyroid rats proved satisfactory and were not affected by the *in vitro* addition of desiccated thyroid.

#### EXPERIMENTAL

*Experiment A*—Ration I was fed to thirteen rats, six of which received in addition 5 mg. of nicotinic acid per 100 gm. of ration. After 2 weeks on this regimen, desiccated thyroid was administered daily for 3 weeks to three rats from each of the two groups. The rats were then sacrificed and the coenzyme I content of the liver and kidney cortex was determined. The results are given in Table II.

The data indicate an appreciable decrease in the coenzyme I content of livers from the thyroid-fed rats. The additional supplementation with 5 mg. of nicotinic acid per 100 gm. of ration had no beneficial effect upon the average coenzyme I content. No significant changes were observed in the coenzyme I concentration of the kidney cortex. It is also apparent that the administration of nicotinic acid had no effect upon the rate of growth of either the control or the thyroid-fed rats.

*Experiment B*—In this experiment, Ration II, identical with Ration I except for its higher content of the synthetic vitamins, was employed in an attempt to determine whether the loss in weight resulting from thyroid administration as observed in Experiment A could be overcome by increasing the level of vitamin

<sup>1</sup> Roche-Organon synthetic crystals.



intake. The effect of increased nicotinic acid consumption upon the low coenzyme I concentration observed in hyperthyroidism was also investigated. Twenty-three rats were divided into the groups shown in Table II. Where indicated, nicotinic acid was

TABLE II  
*Coenzyme I Content of Tissues from Control and Thyroid-Fed Rats*

Ration No. and supplement		No. of rats	Average coenzyme I content,* per gm. fresh weight			Average weekly gain†
			Liver	Kidney cortex	Thigh muscle	
Experiment A						
Control	I	4	γ 1036 (975-1075)	γ 1036 (952-1235)	γ	gm. 15
	I + nicotinic acid‡	3	924 (884-969)	1126 (945-1207)		15
Thyroid-fed	I	3	726 (650-851)	922 (751-1221)		-9
	I + nicotinic acid‡	3	706 (513-997)	1056 (840-1207)		-9
Experiment B						
Control	II	5	1110 (954-1250)	1053 (1000-1141)	663 (550-800)	15
	II + nicotinic acid§	3	929 (875-1000)	1113 (1010-1250)	633 (605-675)	15
Thyroid-fed	II	8	553 (355-722)	666 (462-910)	638 (580-700)	16
	II + nicotinic acid§	7	922 (826-1150)	1013 (819-1341)	659 (600-710)	15

\* The range of values is given in parentheses.

† Computed during the period in which thyroid was being fed.

‡ 5 mg. of nicotinic acid per 100 gm. of ration.

§ 20 mg. of nicotinic acid per 100 gm. of ration.

fed at a level of 20 mg. per 100 gm. of ration. Desiccated thyroid was administered daily over a period of 4 weeks, at the end of which time the coenzyme I concentrations of liver, kidney cortex, and thigh muscle were determined.

Decreases of 50 and 37 per cent were observed in the liver and

kidney cortex, respectively, from the thyroid-fed rats receiving no added nicotinic acid. The coenzyme I concentration of muscle was not affected. It is also evident that supplementation with 20 mg. of nicotinic acid per 100 gm. of ration prevented the decrease in coenzyme I content, although it had no effect upon the growth rate of either the control or the thyroid-fed rats.

*Experiment C*—The observation that the experimentally induced state of hyperthyroidism in the rat resulted in a lowered coenzyme I content of certain tissues prompted a further attempt to produce a true nicotinic acid deficiency in the rat. Twelve weanling rats were placed on the basal Ration III. Six of these rats received in addition 20 mg. of nicotinic acid per 100 gm. of ration. All of the rats were given daily subcutaneous injections of thyroxine. The daily dosage of thyroxine was increased gradually from 0.20 mg. per kilo of body weight until, at the completion of the experiment, a daily dosage of 20 mg. per kilo of body weight was employed. The experiment was continued for 3 months, during which time the rats were weighed weekly and daily food consumption records were taken.

No differences in either growth rate or outward appearance were observed between the control group and the group receiving added nicotinic acid, the average weekly gain for both groups being 20 gm. This weekly growth rate is typical for rats receiving Ration III under normal circumstances and is not increased by the addition of nicotinic acid to the ration. The food consumption of the rats in this series receiving thyroxine was 3 times that normally noted in rats consuming this ration.

#### DISCUSSION

The data presented indicate clearly that the feeding of thyroid to rats receiving a diet low in nicotinic acid results in a marked diminution in the coenzyme I content of both the liver and kidney cortex. It is logical to assume that the increased basal metabolic rate is the cause of this decrease in coenzyme I content. Whether the primary effect of induced hyperthyroidism is to promote a decrease in coenzyme I synthesis or an increase in coenzyme I destruction must remain unanswered for the present. However, it is evident that increased dosages of nicotinic acid can restore the normal coenzyme I content in the hyperthyroid rat, and thus

counteract the deleterious effect of the increased basal metabolic rate.

The above results may assume significance with regard to the nutrition of the hyperthyroid patient. The work of Dann and Kohn (18) and Shourie and Swaminathan (19) has demonstrated that the rat is capable of synthesizing nicotinic acid. It is a recognized fact that the human is limited greatly in his ability to perform such a synthesis. Thus, the occurrence of a decreased coenzyme I concentration observed as a result of the hyperthyroid condition in a species capable of synthesizing nicotinic acid should also be expected, and perhaps to a greater extent, in a species which lacks this ability. It follows that the need for a larger intake of nicotinic acid is indicated in cases of hyperthyroidism. The above reasoning is based upon the supposition that a decreased coenzyme I content in the human may result in a pathological condition. No evidence for this belief can be derived from the rat experiments presented in this paper, since in no case, either in the normal or the hyperthyroid rat, was it possible to demonstrate any beneficial effect of nicotinic acid upon growth. It may be concluded that nicotinic acid was not the factor which limited growth on the experimental diets used and that a decrease in the coenzyme I content of the tissues of the hyperthyroid rat had no effect on growth activity as observed on such rations. A similar situation may not be necessarily true for the human and the negative results obtained with the rat do not establish conclusively the inefficacy of excessive nicotinic acid therapy to humans in a hyperthyroid state.

#### SUMMARY

1. The effect of an induced state of hyperthyroidism upon the coenzyme I content of rat tissues was studied. Marked decreases in the coenzyme I content of the liver and kidney cortex were observed in hyperthyroid rats fed rations low in nicotinic acid. Supplementation of such rations with 20 mg. of nicotinic acid per 100 gm. of ration restored the normal coenzyme I content.

2. On diets low in nicotinic acid, the rates of growth of neither the control nor the hyperthyroid rats were increased by the addition of nicotinic acid.

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# THE EFFECTS OF BIOTIN UPON FAT SYNTHESIS AND METABOLISM

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In previous papers (1, 2) we reported that the administration to rats of an alcohol-soluble fraction of beef liver caused markedly fatty livers, characterized by a high content of cholesterol. Coincidentally there was a gain in body fat and body weight. The fatty livers thus produced differed from the thiamine type of fatty livers in that they were not prevented by supplying choline but were prevented by feeding lipocaic. In a continuation of this work we have found that biotin exerted an action similar to that of the crude liver fraction.

## *Methods*

Rats were employed as test animals. The strain, age, and care were the same as previously reported (3). Basal Diet 1 (3) was used throughout. To deplete the rats of their stores of B vitamins and of fat, they were fed only the basal diet for 3 weeks. At the end of this period body weight and body fat had definitely diminished. During the following week various combinations of supplements were administered to different groups. Thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, choline, and the crystalline biotin solutions were given by subcutaneous injection. Supplements other than those previously mentioned were mixed with the food. Vitamin and choline supplements were given in the following amounts per rat per day, unless otherwise indicated: thiamine hydrochloride (Merck) 25  $\gamma$ , riboflavin (Merck) 20  $\gamma$ , pyridoxine (Merck) 20  $\gamma$ , calcium pantothenate (Merck) 100  $\gamma$ , nicotinic acid (Eastman Kodak Company) 100  $\gamma$ , choline hydrochloride (British Drug Houses), calculated as choline base, 10 mg., biotin (kindly donated by S. M. A. Corpora-

tion) 5  $\gamma$ , lipocaic (generously furnished by Eli Lilly and Company) 300 mg.

The rats were killed by stunning, the livers removed, and total crude fatty acids in the livers and bodies were determined by methods previously published (3, 4). By means of the Schoenheimer and Sperry procedure (5), the total cholesterol in the crude fatty acid fraction of the liver and body was determined. All reported results are averages for groups of ten rats, except for one group in Series 4.

#### *Effect of Biotin upon Fat Synthesis*

*Series 1*—A liver fraction, similar to the one used by us (2), has served as a source of biotin in other laboratories (6–8). It seemed advisable to ascertain whether biotin would produce a fatty liver, not preventable by choline, resembling that caused by the liver fraction. A solution of biotin, prepared in this laboratory from the liver fraction by the method of György, Kuhn, and Lederer (7), and several commercial solutions have been tested. The total crude fatty acids in the liver and body at the end of the experiment are given in Table I. The biotin solutions exerted an effect upon liver fat similar to that of the liver fraction.

*Series 2*—Preliminary experiments showed that biotin would not cause fatty livers unless other B vitamins were supplied. The effect of the known B vitamins upon the activity of biotin was investigated. Acutely fatty livers were not produced unless thiamine, riboflavin, pantothenic acid, and pyridoxine were supplied with biotin. In the absence of thiamine there was no evidence of fat synthesis. An additive effect of the various B vitamins was also evident. The total crude fatty acids in the liver and body are given in Table II.

*Series 3*—The rate of development of fatty livers in rats fed biotin was investigated. The results are given in Table I. Data regarding liver and body cholesterol are also included.

*Series 4*—In the series reported above the biotin solutions were all comparatively crude. In addition to the two mentioned, biotin solutions kindly contributed by Merck and Company, Inc., and by The Fleischmann Laboratories showed similar activity. In all these cases it might be said that the results were not due to biotin but to an impurity contained in the preparations. Through the

TABLE I  
*Effect of Biotin upon Fat and Cholesterol Metabolism*

Series No.	Supplements	Total crude fatty acids		Total cholesterol	
		Liver	Body	Liver	Body
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	None, 3 wks. depletion	2.4	1.7		
	All isolated B vitamins, choline	9.5	6.5		
	Same + liver fraction	17.2	7.7		
	“ + biotin (S. M. A.)	17.8	5.9		
	“ + “ (School of Hygiene)	17.9	6.7		
3	None, 3 wks. depletion	4.6	1.9	0.32	0.15
	All isolated B vitamins, choline, biotin				
	24 hrs.	4.0	2.4	0.22	0.18
	48 “	7.1	3.0	0.34	0.19
	72 “	14.1	4.4	0.50	0.26
	5 days	18.3	6.1	0.80	0.26
	7 “	17.9	7.2	0.93	0.25
4	None, 3 wks. depletion	3.3	1.7	0.38	0.23
	All isolated B vitamins, choline	11.2	7.7	0.71	0.30
	Same + biotin (S. M. A.)	19.3	8.2	1.08	0.27
	“ + “ (crystalline)	17.8	7.3	1.25	0.28
5	All isolated B vitamins, choline	13.2	6.3	0.64	0.29
	Same + biotin (S. M. A.)	13.5	6.5	0.67	0.25
6	None, 3 wks. depletion	3.6	1.9	0.16	0.12
	All isolated B vitamins, choline	6.7	5.7	0.35	0.22
	Same + lipocaiic	4.5	6.3	0.20	0.17
	“ + biotin	15.6	5.9	0.79	0.16
	“ + “ + lipocaiic	3.7	7.8	0.10	0.18

TABLE II  
*Effect of B Vitamins upon Fat Synthesis by Biotin in Series 2*

Biotin.....		+		+	+	+	+	+	+
Thiamine.....		+	+		+	+	+	+	+
Riboflavin.....		+	+	+		+	+	+	+
Ca pantothenate.....		+	+	+	+		+	+	+
Pyridoxine.....		+	+	+	+	+		+	+
Nicotinic acid.....		+	+	+	+	+	+		+
Choline.....		+	+	+	+	+	+	+	
Total crude fatty acids									
Liver, %.....	3.3	18.3	10.7	2.5	5.2	8.6	11.4	21.6	25.5
Body, %.....	1.7	5.6	6.9	1.8	3.6	5.7	5.8	6.1	5.0



courtesy of Dr. du Vigneaud we have been able to test the activity of crystalline biotin. A solution of biotin methyl ester was supplied to us by Dr. du Vigneaud. Because of the limited amount available it was necessary to use only five animals. The biotin solution was injected subcutaneously with a dosage of 5  $\gamma$  per rat per day. It will be observed that the crystalline material had an effect similar to that of the crude solutions.

*Series 5*—It has been reported that raw egg white will inactivate biotin *in vivo* (9). In this series a basal diet consisting of agar 2, salt mixture 4, sucrose 84, cod liver oil concentrate 0.015 parts was used. Each rat received, in addition, 4 cc. of raw egg white per day mixed with the basal diet. Under these conditions the biotin

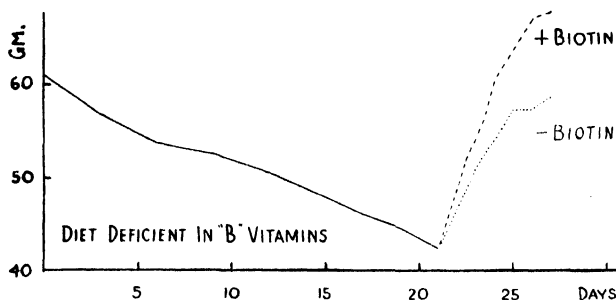


FIG. 1. The effect of biotin upon the body weight. During the supplement period thiamine, choline, riboflavin, pyridoxine, and Ca pantothenate were administered.

solution did not cause an increase in liver fat. We regard this as further evidence that biotin is the active agent in causing this type of fatty liver.

*Series 6*—It has been reported that lipocaic would prevent the development of fatty livers when the beef liver fraction was given (2). Lipocaic was equally effective in preventing the development of fatty livers when biotin was administered, as is shown in this series.

Biotin also resembled the active factor in the liver fraction by causing an increase in body weight, as shown in Fig. 1. A similar liver fraction has been employed as a source of factor W (10). It seems likely that the effect, ascribed to factor W, of causing weight increases might have been due to biotin, acting in conjunc-

tion with the other B vitamins. Other workers (9, 11) have noted the effect of biotin in causing an increase in the body weight of rats.

### *Effect of Biotin upon Cholesterol Metabolism*

In Series 3, when biotin was given, the increase in cholesterol in the liver paralleled the increase in liver fat. Crystalline biotin, as well as the crude biotin solutions, caused the amount of cholesterol in the liver to be greater than in the control group receiving all the isolated B vitamins and choline, as shown in Series 4. Further proof that biotin is concerned in cholesterol metabolism is offered in Series 5, since raw egg white, given with biotin, was able to prevent not only an increase in liver fat but also an increase in liver cholesterol. Lipocaic, which prevented the increase in liver fat caused by biotin, maintained the liver cholesterol at a low level, as shown in Series 6.

### DISCUSSION

Evidence has been presented which indicates that biotin is the active factor, in the alcohol-soluble fraction of beef liver, which causes the amount of fat and cholesterol in the livers of rats to be increased. Final proof that the beef liver fraction owes its activity in producing fatty livers to biotin could only be supplied by the isolation of biotin from the fraction. The following points of evidence are cited to indicate that our assumption is valid: (1) Crude solutions of biotin from several sources exhibited the same physiological action as did the liver fraction. (2) The activity of the crude biotin solutions was obliterated by the simultaneous feeding of egg white. (3) Crystalline biotin had the same physiological action as had the crude solutions of biotin, and as was shown by the liver fraction in producing acutely fatty livers containing large amounts of cholesterol.

Simultaneous administration of lipocaic prevented the increased fat and cholesterol in the liver caused by biotin, while choline was ineffective in this respect. Choline was able to prevent the development of the thiamine type of fatty liver but not the type caused by liver fraction or by biotin. We shall refer to this latter kind of fatty liver as the biotin type to distinguish it from that caused by thiamine. Previous reports have shown that the production of biotin fatty livers can be prevented by lipocaic-like

extracts made from wheat germ and other sources (12) and also by inositol (13).

Biotin produced an acutely fatty liver in a comparatively short time. A significant increase was observed in 24 hours and the maximal level of liver fat was secured in 5 days. It should be made clear that the production of these fatty livers was not due simply to withdrawal of fat from the body. Coincident with the development of fatty livers there was a marked increase in the amount of body fat. In Series 2 the quantity of body fat was increased 2.5 times in 5 days, owing entirely to synthesis, since no fat was furnished in the diet. It seemed possible that the production of the fatty liver by biotin was due to accumulation of cholesterol in the liver. This may be true, but the increase in liver cholesterol did not occur prior to the production of the fatty liver. Biotin did not increase the amount of cholesterol synthesis, but did augment the quantity of cholesterol in the liver.

Engel reported (14) that pantothenic acid caused the same type of fatty liver as that produced by the beef liver fraction. Our results show that choline does not completely prevent the development of fatty livers when thiamine, riboflavin, pyridoxine, and pantothenic acid are supplied. The liver does not, then, contain the large amounts of cholesterol evident when biotin is given. The amount of liver fat can be augmented by giving pantothenic acid in conjunction with thiamine, riboflavin, and pyridoxine, but the amount is much further increased by also supplying biotin. The effect of biotin is not secured unless thiamine, riboflavin, pyridoxine, and pantothenic acid are also furnished. The combined action of these five vitamins is necessary to secure the biotin fatty liver, which completely resembles that produced by the liver fraction.

#### SUMMARY

Impure solutions of biotin from several sources and pure biotin, given to rats in conjunction with thiamine, riboflavin, pyridoxine, and pantothenic acid, caused fatty livers similar to those produced by feeding a fraction from beef liver. The fatty livers were characterized by a high content of cholesterol. The effect of biotin was prevented by simultaneously feeding egg white, lipocaic, or inositol. It is concluded that beef liver fraction owes its activity

to its content of biotin. Biotin had, also, an additive effect upon body weight, similar to that ascribed to factor W.

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# GASOMETRIC DETERMINATION OF CARBOXYL GROUPS IN FREE AMINO ACIDS\*

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## REACTIONS OF AMINO ACIDS WITH NINHYDRIN

The method here presented depends on the fact that  $\alpha$ -amino acids, when boiled in water with an excess of ninhydrin (triketohydrindene hydrate) at pH 1 to 5, evolve the  $\text{CO}_2$  of their carboxyl groups quantitatively in a few minutes. Proline and hydroxyproline yield their carboxyl  $\text{CO}_2$  in the same manner as the amino acids with primary  $\text{NH}_2$  in the  $\alpha$  position. *The reaction is specific for free amino acids in that it requires the presence, in the free, unconjugated state, of both the carboxyl and the neighboring  $\text{NH}_2$  or  $\text{NH}-\text{CH}_2$  Group.* Several reagents other than ninhydrin react in a similar manner, but of those tried, ninhydrin has given the most precise and specific results.

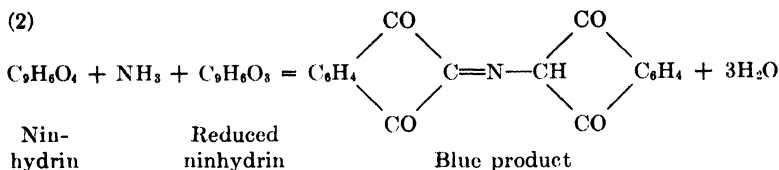
Ruhemann (15), who discovered the reaction of amino acids with ninhydrin, showed qualitatively that monoaminomonocarboxylic acids react with formation of ammonia,  $\text{CO}_2$ , and aldehydes.



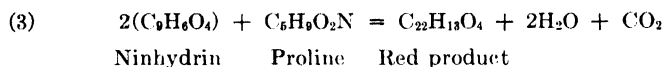
Ruhemann found glycine to be an exception in that it did not form formaldehyde. It does, however, evolve its carboxyl  $\text{CO}_2$ .

If the reaction is carried out at a pH above 4, with amino acids of the type  $\text{R}-\text{CH}(\text{NH}_2) \cdot \text{COOH}$ , a blue color is formed (15) owing to condensation of the liberated ammonia with ninhydrin and its reduction product.

\* This paper gives, with improvements, applications, and experimental basis, a method of which preliminary reports have been presented by two of the authors (24).



With proline and hydroxyproline the reaction is different. As shown by Grassmann and von Arnim (6),  $\text{CO}_2$  is evolved, but not ammonia, and 2 molecules of ninhydrin combine with the decarboxylated residue of the proline or hydroxyproline as follows:



Proline condenses first with 1 molecule of ninhydrin, forming a yellow product (6), then with a 2nd molecule forming a red. When much proline or hydroxyproline is present in amino acid mixtures subjected to the analysis described in this paper, an evanescent yellow color is first seen, later replaced by blue or red. Lysine, on the other hand, forms a black precipitate.

The colored products indicated by Equation 2 do not form at pH below 2.5, but the  $\text{CO}_2$  evolution occurs. The color play with proline and hydroxyproline occurs even at pH 1, but the yellow color persists longer than at pH 2.5 or 4.7, before it gives way to red. At pH 1 the yellow remains until the solution nears the boiling point.

From Equations 1, 2, and 3 it would appear that at least approximate measurements of amino acids in biological fluids could be made by determining the ammonia, the color, the aldehyde, or the  $\text{CO}_2$  formed by reaction with ninhydrin.

As will be shown in another publication by one of the authors (M.), the ammonia can be measured, but under precautions which are not simple.

The color reaction is complicated by the facts that, in mixtures of amino acids, the proportions of red and blue vary, and, more important, that ammonia and various amines other than the amino acids form similar colors with ninhydrin. Harding and MacLean first developed (7) and later condemned (8), because of its lack of specificity, amino acid colorimetry based on the ninhydrin reaction.

Determination of the aldehyde as an analytical measure of

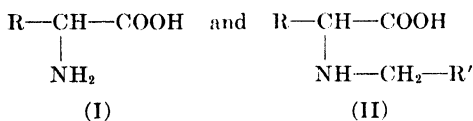
free amino acids formed appeared possible, from results of R. Abderhalden (1), and has recently been used by Virtanen and Laine (29) to determine certain specific amino acids which yield aldehydes that can be determined. Many of the amino acids do not yield such aldehydes, however, and glycine, proline, and hydroxyproline do not form aldehydes.

Of the reaction products formed by amino acids with ninhydrin the  $\text{CO}_2$  appears to offer the most accurate measurement, and the only one which includes all of the amino acids yielded by protein hydrolysis.

Measurement of the evolved  $\text{CO}_2$  was introduced as an analytical procedure by Van Slyke and Dillon (24), in a method of which that published in this paper is a development. Mason (12) applied Van Slyke and Dillon's method to a series of amino acids and peptides, and confirmed its accuracy. Schlager (17) applied it to use in the Warburg manometric apparatus. Christensen, West, and Dimick (2) have also applied Van Slyke and Dillon's conditions for the ninhydrin reaction, and determined the  $\text{CO}_2$  by titration.

The extent to which ninhydrin evolves  $\text{CO}_2$  from the different amino acids and other biological substances has been in part indicated in the preliminary papers of Van Slyke and Dillon (24). A more complete statement is given below, and is followed by the experimental work on which it is based. The results lead to the following generalizations.

*Molecular structures which provide carboxyl groups yielding  $\text{CO}_2$  quantitatively by reaction with ninhydrin under the conditions of the analysis are*



Of Structure I are the amino acids with primary  $\alpha\text{-NH}_2$  groups. Of Structure II are proline, hydroxyproline, and monomethylglycine (sarcosine).

If the  $\text{NH}_2$  group is moved away from the  $\text{COOH}$ , from the  $\alpha$  (Structure I) to the  $\beta$  or  $\gamma$  position, the reactivity of the carboxyl group diminishes but may not disappear. Aspartic acid evolves all the  $\text{CO}_2$  of its  $\beta$ -carboxyl, but  $\beta$ -alanine evolves only 0.16 mole



of  $\text{CO}_2$  at pH 4.7 and no  $\text{CO}_2$  at pH 2.5. In glutamic acid the carboxyl in the  $\gamma$  position to the  $\text{NH}_2$  is almost inert at pH 4.7, quite inert at 2.5.

*The following types of amino acid derivatives yield no  $\text{CO}_2$  under the conditions of the analysis.*

1. Derivatives in which a hydrogen atom of the  $\text{NH}_2$  is replaced by COR, as in *peptides and acetylated or benzoylated amino acids*.

Peptides, except aspartyl and glutamyl peptides with the group  $-\text{C}(\text{NH}_2)\cdot\text{COOH}$  free,<sup>1</sup> accordingly evolve no  $\text{CO}_2$  even from their free carboxyl groups. Hence the *ninhydrin- $\text{CO}_2$  reaction serves to differentiate free amino acids from peptides* more sharply than is possible by methods such as the Sørensen formol titration (19), Van Slyke's nitrous acid method (21), or Willstätter's (30) alcohol titration, which measure the  $\text{NH}_2$  groups of both free amino acids and peptides. Similarly the only previously available method for amino acid carboxyl groups, Zirm and Benedict's (31) modification of Linderstrøm-Lang's (11) "acetone titration," measures the carboxyls of both amino acids and peptides.

2. *Derivatives which have no hydrogen atom on the amino nitrogen.* Monomethylglycine gives off a mole of  $\text{CO}_2$ , whereas dimethylglycine (with both methyl groups on the N) is inert.

3. *Derivatives in which the free carboxyl group is replaced by an ester or acid amide group.* Thus both glycine ester and glycine amide are inert.

4. *Amines, amides, ammonia, and glucosamine* evolve no  $\text{CO}_2$  when boiled with ninhydrin. *Urea*, by itself, gives off  $\text{CO}_2$  slowly (0.01 mole in 5 minutes) when heated at  $100^\circ$  with water at pH 2.5 to 4.7, but in the presence of an excess of ninhydrin even this slow evolution of  $\text{CO}_2$  is retarded, apparently by formation of a relatively stable compound of urea with ninhydrin. Consequently, urea in the amounts present in blood need not be removed before amino acids are determined by the ninhydrin- $\text{CO}_2$  method. In urine the urea-amino acid ratio is so high that it is preferable to remove the urea.

Simple organic acids such as acetic, and hydroxy acids such as lactic and citric evolve no  $\text{CO}_2$ .

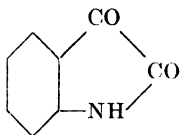
<sup>1</sup> Glutathione is such a peptide, since the  $-\text{CH}(\text{NH}_2)\cdot\text{COOH}$  group of the glutamyl radical is free. Such peptides do not appear to occur in important amounts in a protein digest (see Fig. 6).

*Keto acids*, such as pyruvic and acetoacetic, decompose with loss of  $\text{CO}_2$  when boiled with water; they can be completely decomposed, however, by preliminary boiling so as not to interfere with measurement of amino acid carboxyl  $\text{CO}_2$ .

*Ascorbic acid* evolves about 0.1 mole of  $\text{CO}_2$  in the time required for complete reaction by the amino acids. The amounts of ascorbic acid present in body fluids and excretions are too slight, however, to affect amino acid determinations significantly.

*Other Reagents for Evolution of Carboxyl  $\text{CO}_2$  from Amino Acids*

The ability to evolve the carboxyl  $\text{CO}_2$  from amino acids is not peculiar to ninhydrin. Isatin, in structure similar to ninhydrin,



was shown by Grassmann and von Arnim (6) to react similarly with amino acids in glacial acetic acid solution, evolving  $\text{CO}_2$  and forming colored products. We have found that amino acids boiled with isatin in glacial acetic acid give theoretical yields of  $\text{CO}_2$ ; but the reaction is slower, and isatin as a reagent has no advantage over ninhydrin except lower cost. Isatin is too insoluble in water to apply in aqueous solutions.

Recently Schiller (16) has suggested sodium  $\beta$ -naphthoquinone sulfonate, used in the colorimetric method of Folin (5), as an economical substitute for ninhydrin in our preliminary method (24). We have confirmed Schiller in the fact that the naphthoquinone evolves  $\text{CO}_2$  from amino acids; but we find that the quinone itself also decomposes in hot aqueous solution to yield  $\text{CO}_2$ . This spontaneous decomposition of the reagent causes so high and variable a blank that the error of the amino acid determination is much greater than by the ninhydrin method.

Chloramine-T (the sodium salt of *p*-toluenesulfonchloramide,  $\text{CH}_3 \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_2 \cdot \text{NH} \cdot \text{Cl}$ ) was shown by Dakin (4) to cause at 20–50° evolution of  $\text{CO}_2$ , 1 mole from some of the monocarboxylic acids, 2 moles from aspartic acid. In quantitative studies of the reaction with the Barcroft-Warburg apparatus by Cohen (3) and by Krebs (quoted by Cohen) it was found that most of the mono-

amino acids yielded from 1.01 to 1.15 moles of  $\text{CO}_2$  at pH 4.7, while arginine, glycine, and lysine yielded over 1.2 moles, and aspartic acid yielded 2 moles. It is evident from the data of Krebs and Cohen that the results with several of the amino acids are not even approximately quantitative. This conclusion we have confirmed, but we have found nevertheless that with the Van Slyke-Neill manometric apparatus and properly chosen pH one can obtain theoretical results with some of the amino acids, and within 2 per cent of theoretical with most of them, exceptions being cystine, tyrosine, glycine, and tryptophane, with which the errors are greater. With peptides, chloramine-T is not quite as inert as ninhydrin, yielding 0.01 to 0.05 mole of  $\text{CO}_2$  per mole of peptide. For analyses of certain mixtures of amino acids chloramine-T can be used as a substitute for ninhydrin, and has the advantage of low cost and low reacting temperature. For general analyses of amino acid mixtures and biological fluids, however, it does not give results as exact as ninhydrin.

#### APPARATUS

*Van Slyke-Neill manometric apparatus* (14, 26).

*A storage vessel for  $\text{CO}_2$ -free 0.5 N sodium hydroxide.* The tube with soda lime protection, shown in Fig. 3, D of the paper by Van Slyke and Folch (25).

*Alundum pieces* for smooth boiling (p. 511, Van Slyke and Folch (25)).

*Reaction vessels.* For the reaction with ninhydrin a vessel of 15 to 25 cc. capacity is needed, capable of holding a vacuum and of being heated in boiling water. It is also essential that there should be but little rubber surface exposed to the gases in the chamber, as  $\text{CO}_2$  diffuses both into and out of rubber.<sup>2</sup> Three reaction vessels that have proved convenient and have given accurate results are

<sup>2</sup> Black rubber stoppers bearing glass outlet tubes of 6 mm. diameter were tried in place of the adapters shown in Fig. 1, A, but the stoppers, with their larger exposure of rubber surface, absorbed appreciable amounts of  $\text{CO}_2$  when the reaction vessels were permitted to stand closed for some time after removal from the bath. The results obtained in a typical series of analyses, in which vessels with rubber stoppers were used, serve as an example. Known amounts of alanine were boiled in several reaction vessels with stoppers at one time and then cooled and analyzed for  $\text{CO}_2$  at varying intervals thereafter. Immediate analysis gave 99.7 per cent of theoretical;

shown in Fig. 1. The vessel in Fig. 1, *A* is the type we have used most.<sup>3</sup> It consists of two parts, a test-tube of 21 mm. diameter in its wider part, narrowed to 16 mm. at the top, and an adapter which serves to close the tube with minimal exposure of rubber surface. The tube and adapter are both of Pyrex glass, 1 to 1.5 mm. thick.

The adapter bears two rubber connecting tubes which remain routinely attached to it, forming one permanent piece. Both rubber tubes have walls  $\frac{3}{16}$  inch thick. The upper piece is of  $\frac{1}{8}$  inch bore and the lower of  $\frac{1}{2}$  inch bore. It is essential that the rubber in both be "of minimal sulfur content."<sup>4</sup> Ordinary rubber gives high blank values, perhaps because of evolution of traces of hydrogen sulfide. Before the rubber connections are used, they are scrubbed out with a test-tube brush, digested on a steam bath for half an hour with 2 N sodium hydroxide, then boiled for a few minutes in dilute hydrochloric acid, and rinsed with water. When the proper rubber is used and cleaned in this manner, it does not affect the accuracy of analyses even when precision is of the order of 2 parts per 1000.

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analysis after 25 minutes, 98.5 per cent; analysis after 50 minutes, 97.4 per cent. Peculiarly enough, the absorption of  $\text{CO}_2$  was but slight during the 6 minutes of heating in the bath. When stoppers that had absorbed  $\text{CO}_2$  were used later in blank analyses, they gave slightly high values, owing apparently to diffusion out into the vessels of  $\text{CO}_2$  that the stoppers had previously absorbed. Reaction vessels arranged with adapters, as shown in Fig. 1, *A* and *B*, instead of stoppers showed no evidence of loss of  $\text{CO}_2$  when permitted to stand for 2 hours between completion of the reaction and determination of the  $\text{CO}_2$ .

<sup>3</sup> The tube and adapter (Fig. 1, *A*) are carried in stock by the Corning Glass Works (No. 9535, Catalogue LP21, 1941), and can be obtained also from makers of accessories of the Van Slyke-Neill manometric apparatus. The all-glass reaction vessel, Fig. 1, *C*, is made by the Hopf Glass Apparatus Company, 192 Third Avenue, New York.

<sup>4</sup> We have used the black rubber tubing listed as of "minimal sulfur content," No. 30691 of Eimer and Amend's 1936 catalogue. After use for about 3 months the repeated steamings cause enough loss of elasticity to permit slow leakage of air into the evacuated vessel, and fresh rubber tubing must be installed on the adapters. The rubber ultimately deteriorates even without use, merely by standing in laboratory air. *The first signs of deterioration are the occurrence of carboxyl results 1 or 2 per cent too low, and the failure of duplicates to check as exactly as usual.* When these occur, renew the rubber connections.

Vessel *B* (Fig. 1) is identical with *A*, except that a 25 cc. Pyrex Erlenmeyer flask is substituted for the special reaction tube. A stock flask with a neck of 15 to 17 mm. outer diameter and but a slight flange will serve. If the flange is too wide, it can be ground off or softened in a blast flame and pushed in. Vessel *B* lacks the

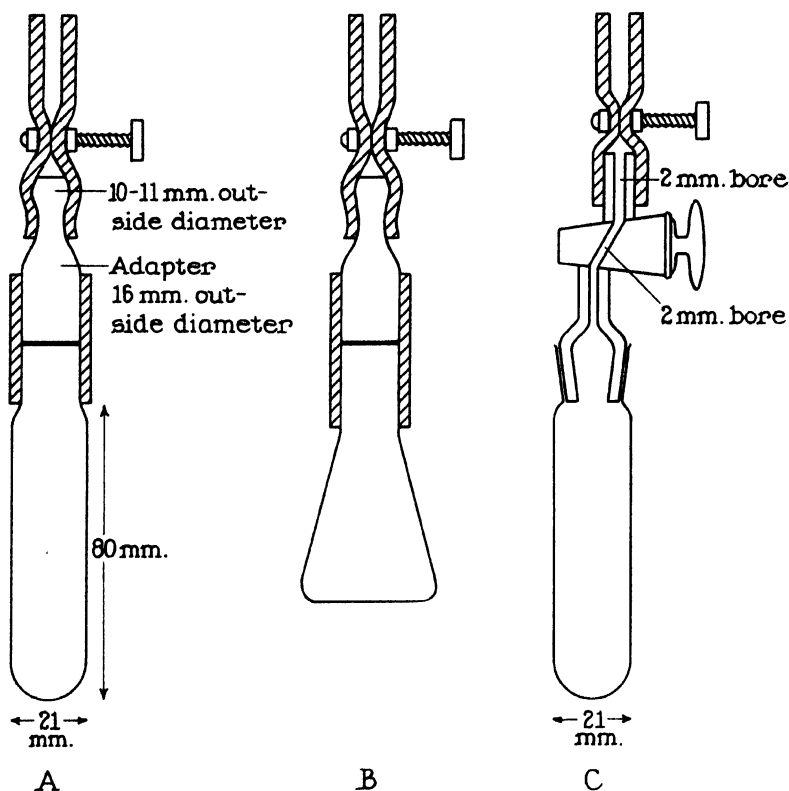


FIG. 1. Reaction vessels of Pyrex glass

convenience of fitting into a test-tube rack, but it can be prepared in any laboratory, and a wire basket can be used in place of the rack to boil a number together.

Vessel *C* provides an all-glass container. To hold a tight vacuum in the boiling water bath and for the varying periods that are likely to intervene afterwards before the  $\text{CO}_2$  is determined, the

usual stock uniform ground joints do not suffice. The joint and the glass cock of each tube must be especially ground, as in vacuum desiccators, to hold a complete vacuum. The three glass parts are all of Pyrex, in order to insure uniform expansion when heated. Both the cock and the glass joint are lubricated with glycerol each time the vessel is used.<sup>5</sup>

When the carboxyl method is used as a routine analytical procedure, the most convenient reaction vessel will be *A*, with *B* as an alternative. When only an occasional determination is to be done, the all-glass vessel *C* is desirable, because its only rubber part does not come into contact with the gases during the reaction, and if the rubber deteriorates somewhat it is not so important. For routine analyses *A* is preferable, because it is cheaper, has no joints requiring lubrication, and the adapters fit interchangeably on all the tubes. With regard to accuracy, *A* and *B*, when the rubber connections are as directed, are quite equal to the all-glass *C*.

If, as may rarely occur, it is desirable to analyze amino acid solutions of more than 5 cc. volume, a 50 cc. Erlenmeyer arranged as in Fig. 1, *B* is preferable to the usual 20 to 25 cc. reaction vessel.

*Heavy metal clamps.* The clamps shown in Fig. 1, *A* and *B* serve both to close the vessels and to sink them below the water during boiling. They should therefore weigh over 20 gm. One may use heavy screw clamps, as shown in the figure, but more convenient are "heavy duty, castalloy" cylindrical pinch clamps.<sup>6</sup>

*Calibrated glass spoons for measuring ninhydrin and citrate buffer.* These may be made as shown in Fig. 4 of Van Slyke and Folch (25). It is convenient to prepare four such spoons, to hold 50 and 100 mg. of ninhydrin, and 50 and 100 mg. of buffer. The 50 mg. spoons are made of tubing of 3.5 to 4 mm. bore, and the 100

<sup>5</sup> The ground joint is lubricated, after the ninhydrin is placed in the vessel, by drawing a ring of glycerol about the ground inner surface of the reaction tube, before the stopper is inserted. When the vessel is removed from the boiling water bath at the end of the reaction, another ring of glycerol is drawn about the rim of the joint; this glycerol seeps into the joint, where it replaces lubricant lost during the boiling, and prevents leakage of air into the vessel while the latter awaits the CO<sub>2</sub> determination.

<sup>6</sup> The clamps under this name are obtainable from the Fisher Scientific Company of Pittsburgh.

mg. spoons of tubing of about 5 mm. bore. The amounts of ninhydrin must be measurable within  $\pm 10$  per cent accuracy, for the boiling periods of the reaction depend on the ninhydrin concentration.

*Water bath for heating reaction vessels.* To insure complete reaction of the amino acids with ninhydrin in the time allowed, it is essential that the reacting solution be raised to 99–100° within 2 minutes after immersion in the bath. The volume of boiling water in the bath must be so great that when the cold reaction vessels are immersed in it the temperature of the bath does not fall below 98°, and the flame under the bath must be large enough to start the boiling again in a few seconds.

The most convenient form of bath for multiple analyses is a cylindrical pot of non-rusting sheet metal about 8 inches in diameter and 8 or 10 inches deep, provided with a thermometer, a loose fitting cover, and an inflow-outflow attachment to keep the boiling water at constant level.<sup>7</sup> Sheet metal is preferable to enameled ware because the metal bath heats much more rapidly.

If only one to three reaction vessels are to be boiled at a time, one may use a 2 liter Pyrex beaker as bath.

*Rack for reaction vessels.* For multiple analyses it is convenient to have metal racks capable of holding several reaction vessels in the bath.<sup>8</sup>

#### REAGENTS

✓ *Ninhydrin.* The ninhydrin may be prepared from ethyl phthalate as described by Teeters and Shriner (20). We have used preparations made by the Eastman Kodak Company. Each lot is ground to a fine powder and stored in dark glass.

✓ *Citrate buffers.* For the ninhydrin reaction in aqueous medium two citrate buffers are used. One, for pH 4.7, consists of a mixture

<sup>7</sup> The water level regulator attachments are listed as No. 17340 in Eimer and Amend's catalogue, and as No. 9876 in the catalogue of the Arthur H. Thomas Company (1931).

<sup>8</sup> Cylindrical test-tube racks of non-rusting metal, 6 inches in diameter, capable of carrying test-tubes of 22 mm. ( $\frac{7}{8}$  inch) diameter, are listed as No. 32003 in Eimer and Amend's catalogue (1936) and No. 9487 in the catalogue of the Arthur H. Thomas Company (1931). These racks fit the tubular reaction vessels (Fig. 1, *A* and *C*). If Erlenmeyer flasks are used (Fig. 1, *B*) as reaction vessels, a wire basket divided into sections with two sets of cross wires serves as a rack.

of 17.65 gm. of trisodium citrate,  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ , and 8.40 gm. of citric acid,  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ . The other, for pH 2.5, consists of 2.06 gm. of trisodium citrate and 19.15 gm. of citric acid. In preparing the buffers the trisodium citrate and citric acid are both finely ground, separately, in an agate mortar and are then mixed in correct proportions. After mixing, they cake appreciably, but the cake is easily broken up by two subsequent grindings in the mortar. Thereafter, they remain powdered. Both buffers are used in the solid form. They should be free of  $\text{CO}_2$  when tested in blank analyses.

*6 M  $\text{H}_3\text{PO}_4$ .* Syrupy phosphoric acid of 1.72 specific gravity is mixed with 1.5 volumes of water to make approximately 6.0 M  $\text{H}_3\text{PO}_4$ . The solution is tested by diluting a portion 100-fold and titrating aliquots with 0.1 N alkali to the full red color of phenolphthalein. The 100-fold dilute phosphoric acid should titrate as 0.12 N. To the approximately 6 M stock solution syrupy phosphoric acid or water in proportions calculated from the titration is added to bring the concentration to  $6.0 \pm 0.1$  M. This solution is used when the ninhydrin reaction is run at pH approximately 1; 0.2 cc. of the 6.0 M  $\text{H}_3\text{PO}_4$  added to 1 cc. of water, forming 1 M  $\text{H}_3\text{PO}_4$  with pH slightly under 1, when no amino acid is present. The amino acid in the sample raises the pH slightly; for the purposes of the analyses it is sufficiently constant and near to 1.

*Approximately 0.5 N NaOH of minimal  $\text{CO}_2$  content.* Solid NaOH is dissolved in an equal weight of water and the solution is let stand until the carbonate settles. The concentrated solution is standardized by pipetting 7 cc. from a graduated pipette into water in a 250 cc. flask, diluting to volume, and titrating against standard acid. From the result one estimates how much more or less than 7 cc. of the concentrated alkali is required to make 0.5 N when diluted to 250 cc. To prepare the reagent one fills a 250 cc. flask within about 10 cc. of the mark with distilled water, in order to displace the air and its  $\text{CO}_2$ . The estimated volume of concentrated NaOH solution is run into the water from a graduated pipette dipping beneath the surface. A few drops of 1 per cent alizarin sulfonate indicator solution are added, and the flask is filled to the mark, closed, and shaken. The solution is drawn directly from the flask to a storage vessel (Fig. 3, D of Van Slyke and Folch (25)) which provides protection from atmospheric  $\text{CO}_2$ . The  $\text{CO}_2$  content of a solution thus prepared is such that 2 cc. of



the solution yield enough  $\text{CO}_2$  to exert about 6 mm. of pressure at 2 cc. in blank analyses, indicating a  $\text{CO}_2$  concentration of about 0.0003 M.

*Approximately 5 N NaOH.* This is prepared from the concentrated 1:1 NaOH solution by diluting 1 volume with 3 volumes of water.

*Approximately 2 N lactic acid.* 2 volumes of concentrated lactic acid (sp. gr. 1.20) are diluted with water to 10 volumes.

#### PROCEDURE FOR SUBMICRO- AND MICROANALYSES

✓ *Size of Samples for Submicro-, Micro-, and Macroanalyses—* Table I shows the range of samples for each type of analysis, and

TABLE I  
*Range of Submicro-, Micro-, and Macroanalyses*

Type of analysis	Volume at which $\text{PCO}_2$ is measured	Range of sample to give $\text{PCO}_2$ of 100–500 mm.	
		Carboxyl carbon	Carboxyl nitrogen*
	cc.	mg.	mg.
Submicro	0.5	0.035–0.18	0.04–0.21
Micro	2.0	0.14–0.7	0.2–0.8
Macro	10.0	0.7–3.5	0.8–4.1

\* Since, in calculations involving amino acids, nitrogen values are more often used than carbon, the term "*carboxyl nitrogen*," or "*COOH-N*" will be employed to indicate  $\alpha$ -nitrogen values estimated as 1 atom of nitrogen per molecule of carboxyl  $\text{CO}_2$  evolved in the ninhydrin- $\text{CO}_2$  analysis. This estimation is valid for all the amino acids except aspartic. "Carboxyl nitrogen" is calculated as carboxyl carbon  $\times 14/12$ .

serves to define "submicro," "micro," and "macro," as the terms are applied in the following pages.

For analyses of blood and urine, where minimal amounts of amino acid are determined, the submicroanalysis is used.

For most other work the microanalysis will be used. It is, for example, the one that fits samples of 4 to 6 mg. of amino acid weighed on the micro balance, and suffices to keep the mean error within  $\pm 2$  parts per 1000. Both the above analyses are performed with the apparatus shown in Fig. 3, marked for measurement of gas pressures at 2 and 0.5 cc. volume.

The macroanalysis requires a chamber of the type used for combustions (25, 27), calibrated for measurements with the gas at

10 cc. volume. It will hardly be required unless one desires to use samples weighed on a macro balance.

*Preparation of Samples for Analysis*—If a dry amino acid is analyzed, the sample, usually 4 to 8 mg. for a "micro" analysis, is weighed in the counterbalanced aluminum scoop<sup>9</sup> shown in Fig. 5 of Van Slyke and Folch (25), is placed in one of the reaction vessels shown in Fig. 1, and is dissolved in a measured volume of water, 1 to 5 cc. (2 cc. are usually convenient).

If the amino acids are already in solution, a sample of 1 to 5 cc. is placed in one of the reaction vessels of Fig. 1.

\* *Adjustment of pH*—By reference to Table IV and the discussion on p. 654 under "Choice of pH. . ." one can decide whether pH 1, 2.5, or 4.7 is preferable for the material to be analyzed. For general analyses of biological material pH 2.5 will generally be used. If no significant amounts of free acid or alkali or buffers other than the amino acids themselves are present, the sample is brought to the desired pH by the following additions: For pH 2.5 or 4.7, 50 mg. of citrate buffer when the sample volume is 1 or 2 cc., 100 mg. when the volume is 3 to 5 cc. For pH 1, 6 M  $\text{H}_3\text{PO}_4$  is added in the proportion of 0.2 cc. for each cc. of sample.

If significant amounts of buffer or of free acid or alkali are present in the solution to be analyzed, a drop of 0.1 per cent brom-phenol blue or other indicator changing color at pH 3 to 4 is added, and the solution is brought just to the acid side of the indicator. Citrate buffer or phosphoric acid is then added. As a rule this preliminary adjustment is not needed.

*Removal of Preformed  $\text{CO}_2$* —A few pieces of alundum are added to prevent bumping, and a drop of caprylic alcohol to prevent foam if protein or other foam-producing material is present. The reaction vessel is then boiled over a small flame vigorously for 20 to 30 seconds. If there is reason to suspect the presence of  $\alpha$ -keto acids or other unstable organic compounds capable of evolving  $\text{CO}_2$  merely by heating to 100°, either the boiling, or heating in a

<sup>9</sup> In the original description of the use of the scoop (25) directions were given to let the scoop rest in the balance case for 3 minutes after the sample has been dumped out of it, to allow the scoop to return to balance temperature before it was weighed. It has since been found that this 3 minute wait is usually unnecessary if the reaction vessel into which the sample is dumped is held in a test-tube holder instead of in the hand.

water bath, is continued as long as may be necessary to complete the evolution of such  $\text{CO}_2$ .

If a submicroanalysis is done, or a microanalysis with more than 2 cc. of solution in the reaction vessel, the vessel is tightly stoppered as soon as the preformed  $\text{CO}_2$  is boiled off, and is kept stoppered until the ninhydrin is added.

This precaution is to prevent saturation of the solution with atmospheric  $\text{CO}_2$  as the solution cools. The extent to which the saturation could increase the apparent carboxyl  $\text{CO}_2$  is proportional to the volume of the solution. The maximum volume of  $\text{CO}_2$  absorbed by 5 cc. of solution saturated with atmospheric air of normal (0.03 per cent)  $\text{CO}_2$  content is about 1.5 c.mm., which would increase the manometric  $P_{\text{CO}_2}$  by about 2.5 mm., measured with the gas volume at 0.5 cc. Laboratory air in winter might at times triple this. In macroanalyses, or in microanalyses with 2 cc. or less of solution, the error from reabsorbed atmospheric  $\text{CO}_2$  would not be significant, and it is not necessary to stopper the reaction vessels after boiling off preformed  $\text{CO}_2$ .

*Chilling the Solution*—After the preformed  $\text{CO}_2$  has been removed, the solution is cooled to below  $20^\circ$  if the solution is at pH 4.7. Below  $25^\circ$  suffices if the pH is 2.5 or 1. The precaution of chilling before adding the ninhydrin is necessary to prevent the reaction between ninhydrin and  $\text{CO}_2$  from evolving significant amounts of  $\text{CO}_2$  to be lost when the air is evacuated in the next step of the analysis. If the solution is cooled as directed, and the evacuation and closing of the vessel are completed within 60 seconds after the ninhydrin is added, no significant amount of  $\text{CO}_2$  is lost. If the solution is cooled below  $10^\circ$  (3 minutes in ice water) before ninhydrin is added, one may take several minutes for evacuation without loss of  $\text{CO}_2$ .

The rates of  $\text{CO}_2$  formation from alanine in solutions containing 50 mg. of ninhydrin per cc. were determined over periods up to 30 minutes, and found to be as follows, in terms of percentages of total theoretical  $\text{CO}_2$  formed *per minute*:

At pH 2.5,	0.03%	at $20^\circ$ ,	0.06%	at $30^\circ$
“ “ 4.7,	0.3%	“ $20^\circ$ ,	0.6%	“ $30^\circ$

It is notable that at room temperature the reaction is 10 times as fast at pH 4.7 as at 2.5. (At  $100^\circ$  pH change over this range has a relatively small effect (see Fig. 2).)

The margin of safety for evacuating and closing the reaction vessel during the 1st minute is somewhat greater than is indicated by the above

velocity figures, because the ninhydrin dissolves rather slowly at room temperature. In the first 2 minutes after addition of the ninhydrin, even when the mixture was shaken to accelerate dissolving, the rates of  $\text{CO}_2$  evolution noted were less than half the mean rates recorded above for the 30 minute period.

*Addition of Ninhydrin and Evacuation of Vessel*—The detached adapter (Fig. 1, *A* or *B*) or glass stopper (Fig. 1, *C*) is made ready, so that no time will be lost in closing and evacuating the reaction vessel after the ninhydrin is added. The wide rubber tube of the adapter is dipped into water to lubricate it for slipping over the neck of the vessel, and the narrow rubber tube, with clamp in place but not closed, is connected to a good suction pump provided with a manometer. If the glass-stoppered vessel (Fig. 1, *C*) is used, the stopper is connected with the pump.<sup>5</sup>

To the chilled solution in the reaction vessel one then adds the ninhydrin, measured within  $\pm 10$  per cent from one of the glass spoons described under "Apparatus." The amount of ninhydrin added is usually 50 mg. if the volume of solution is 1 or 2 cc., 100 mg. if the volume is 3 to 5 cc.

As soon as the ninhydrin is added, the adapter or glass stopper is fitted to the reaction vessel, and the latter is evacuated and closed with the clamp as quickly as possible. With a good water pump connected directly without an intervening "safety" flask, evacuation to 20 or 30 mm. pressure requires only 10 seconds.

If a pump is not available, the reaction vessel can be evacuated by attaching it to the capillary outlet of the mercury-filled Van Slyke-Neill 50 cc. chamber, and thrice lowering the mercury to the bottom of the chamber and ejecting the gases that are drawn over from the vessel. As this takes more time than evacuation with a good pump, it is well to have the solution in the vessel cooled below  $15^\circ$  to prevent loss of traces of carboxyl  $\text{CO}_2$ .

When several analyses are to be done, it is convenient first to charge and evacuate all the reaction vessels so that they can be subsequently boiled together. Each vessel must, however, be evacuated by itself immediately after the ninhydrin has been added.

The preliminary evacuation, which has been introduced since the original notes on the method (24) were published, serves three purposes.

1. *Removal of atmospheric  $\text{CO}_2$  as a source of error.* If the laboratory air

were pure atmosphere with a constant 0.03 per cent of  $\text{CO}_2$ , the  $\text{CO}_2$  in the approximately 25 cc. contained in the reaction vessel would add about 3 mm. to the blanks when pressures are read with the gas at 2 cc. volume, and 12 mm. when read at 0.5 cc. volume. In practice the air in New York, especially in winter, is likely to contain 2 or 3 times this amount of  $\text{CO}_2$  and, more important, the  $\text{CO}_2$  content may vary from hour to hour. By removing the air, variation in the atmospheric  $\text{CO}_2$  as a source of error is eliminated. For the submicroanalyses in which pressures are read with the gas at 0.5 cc. volume, this elimination is a necessity for results accurate within 1 per cent.

2. *Removal of inert gases to accelerate  $\text{CO}_2$  absorption.* After the reaction with ninhydrin has been completed, the transfer of  $\text{CO}_2$  from the reaction vessel to the alkali solution in the manometric chamber is quicker in the absence of the inert gases of the air. If the original air content of the vessel were present in the system during the transfer, the air molecules would so retard the contacts of  $\text{CO}_2$  with the alkali in the chamber that 3 times as many passages of the gas back and forth would be necessary to get all the  $\text{CO}_2$  absorbed.

3. *Prevention of positive pressure.* The evacuated tube can be closed and heated in a water bath without developing enough positive pressure to cause danger of losing  $\text{CO}_2$ . In consequence a number of tubes can be heated together, and their  $\text{CO}_2$  contents determined in rapid sequence later.

*Heating with Ninhydrin*—The reaction vessels are placed in a rack, and are completely immersed in an upright position in a bath of *actively* boiling water. Immersion in the boiling water is continued for a period which depends on the ninhydrin concentration and pH of the solution, as shown in Fig. 2.<sup>10</sup> After the first minute of heating the vessels are shaken a little, without removal from the bath, to insure distribution of the dissolved ninhydrin. The vessels must be immersed as far as tie clamps.

It is desirable, particularly when determining amino acids in complex biological mixtures, to limit the boiling time accurately to the specified number of minutes. Lysine, cystine, and glutamic acid give more than the theoretical yield of  $\text{CO}_2$  if the boiling with ninhydrin is unduly prolonged, and peptides and proteins are

<sup>10</sup> During the heating of an amino acid at  $100^\circ$  with the excess of ninhydrin used, the reaction follows approximately a linear curve, when the log of unevolved  $\text{CO}_2$  is plotted against minutes of heating. The points on the curves of Fig. 2 were obtained with alanine by plotting such logarithmic curves until they indicated evolution of 99.9 per cent of the theoretical  $\text{CO}_2$ . In so far as the amino acids differ, alanine is one of the more slowly reacting ones, and the periods indicated are adequate for all.

very slowly hydrolyzed. Error due to these causes is minimal if the heating is limited to the specified durations.

*Absorption of  $\text{CO}_2$  by Alkali in Van Slyke-Neill Chamber*—The chamber is prepared by measuring into it 2 cc. of the 0.5 N sodium hydroxide solution, as described on p. 521 of the paper by Van Slyke and Folch (25). (The alkali used contains no hydrazine

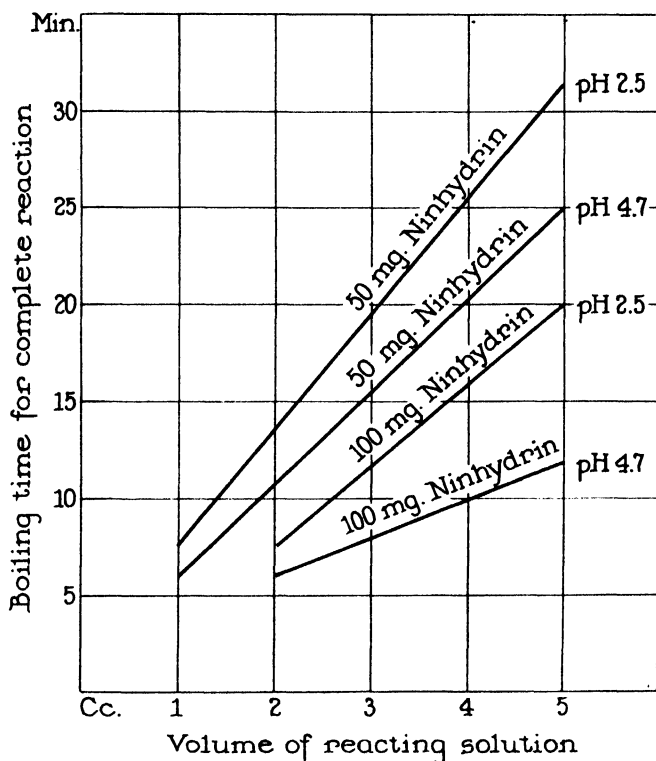


FIG. 2. Boiling periods required to complete the formation of  $\text{CO}_2$  in solutions of pH 2.5 and 4.7 and varying ninhydrin concentrations.

in the present analysis.) The reaction vessel is brought to 38–40°<sup>11</sup> and is then attached to the manometric chamber, as shown

<sup>11</sup> A 2 liter beaker of water with a micro burner several inches below can easily be kept at this temperature, and serves as a convenient bath for warming the tubes. When  $\text{CO}_2$  determinations are run off on a series of reaction vessels which have been boiled together, one vessel at a time is

in Fig. 3. The mercury in the chamber is lowered to the middle of the chamber (Fig. 3 shows the relations at this moment), and

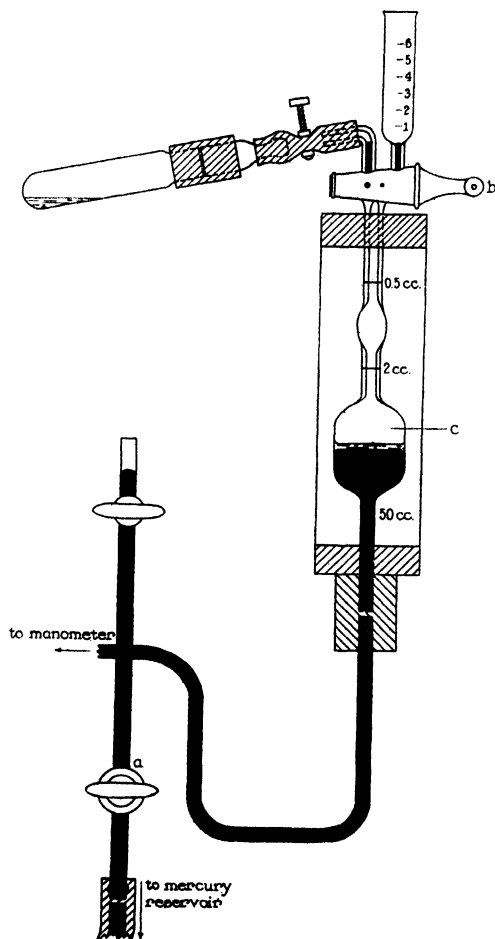


FIG. 3. Manometric chamber with reaction vessel attached, but not yet connected with interior of chamber.

chamber and flask are connected by removing the clamp and turning the stop-cock at the top of the chamber. The connecting

placed in the 38–40° bath, and each is allowed to warm for about 10 minutes while the CO<sub>2</sub> is determined in its predecessor.

rubber tube is inspected to make sure that it opened when the clamp was removed. The glass cock at the top of the chamber is also inspected to make sure that it is completely open, so that gas flow through it will be free and unhindered.

The  $\text{CO}_2$  is now transferred from the warm reaction vessel to the alkali in the chamber by raising and lowering the mercury in the chamber, making each round trip in about 10 seconds. *During each lowering the reaction vessel is shaken by hand*, so that the solution in it whirls about the wall. The number of excursions of the mercury required to obtain complete absorption of the  $\text{CO}_2$  depends somewhat on the volume of the solution. With 1 cc. of solution six excursions suffice; with 2 cc., seven; with 3 cc., eight; with 4 cc., nine; with 5 cc., ten.

The solution in the reaction vessel *must be warmed to 38–40° at the beginning of the extraction* and must be *shaken each time the mercury is lowered*, or the transfer of  $\text{CO}_2$  will not be complete with the above number of excursions. If the extraction starts with the solution at 20° instead of 38°, 3 times as many excursions are required. Also, even if the solution is warmed to 38°, but is not shaken during the excursions, about 3 times as many are required. It is not desirable to start the extraction with the solution warmer than 40°, or an appreciable volume of water may distil over from the reaction vessel into the chamber, and cause a slightly low result by making the *S* volume greater than the 3 cc. for which the factors are calculated.

The labor of raising and lowering the mercury can be avoided by the use of a suction pump, as described on p. 524 and in Fig. 4 of Van Slyke and Folch (25). Because of the high vacuum in the manometric chamber in the present analyses, however, it is desirable to replace the 2 mm. opening in Fig. 4, *B* of Van Slyke and Folch by a capillary of 1 mm. bore and 3 to 5 cm. in length. This serves as a brake to the intruding air when the suction on the leveling bulb is released, and prevents the mercury in the manometric chamber from rising too fast to control. Also, to augment the braking action, it is well to station the mercury leveling bulb 8 or 10 inches below the 50 cc. mark on the chamber. The most convenient means of closing and opening the capillary aperture of the device is a flap of chamois skin, alternately laid on and removed from the opening. If many series of analyses are done, this simple suction pump device will be found highly desirable.

After the last upward excursion the mercury is lowered to the middle of the chamber, and both the cock at the top and the cock



leading to the leveling bulb are closed. The reaction vessel is then removed, and the capillary by which it has been attached is sealed with mercury, as shown in Figure 3, A of Van Slyke and Folch (25).

*Determination of CO<sub>2</sub> in the Manometric Apparatus*—From this point the technique is that described for the same phases of the carbon determination by Van Slyke and Folch (25) under the headings, "Ejection of unabsorbed gases," "Extraction of CO<sub>2</sub> and reading of  $p_1$ ," "Reabsorption of CO<sub>2</sub> and reading of  $p_2$ ," and "Washing chamber after analysis" (see pp. 524–527 of their paper (25)).

The manometer readings are made with the gas volume at 0.5 or 2.0 cc., according to the amount of amino acid carboxyl, as indicated in Table I.

#### *Blank Analysis for c Correction*

The  $c$  correction, due chiefly to carbonate in the 0.5 N NaOH, is the value of  $p_1 - p_2$  found in a blank analysis, with the reagents present, but no amino acid. The correction must be redetermined whenever fresh 0.5 N NaOH is prepared. The ninhydrin may be omitted from the blank analysis, since in no case has it been found to contribute to the  $c$  correction. Omission of the ninhydrin from the blanks results in an appreciable saving of this expensive reagent. However, before one relies on blanks in which ninhydrin is omitted, its lack of effect should be confirmed with the particular reagents used. The value of  $c$ , with properly prepared 0.5 N NaOH, is usually 6 or 7 mm., measured at 2 cc. volume, or 25 to 30 mm. at 0.5 cc. volume, and is constant for a given set of reagents.

#### PROCEDURE FOR MACROANALYSES

For samples with 1.8 to 4.0 mg. of carboxyl nitrogen, the procedure is identical with that for smaller samples, except in the following details.

The minimum volume of solution used is 2 instead of 1 cc.

The amounts of ninhydrin and citrate buffer are increased to 150 mg. of ninhydrin and 200 mg. of citrate.

The CO<sub>2</sub> pressure is measured with the gas at 10 cc. volume.

A drop of caprylic alcohol is routinely added to the reaction vessel, because so much precipitate is likely to form in it that

foaming will occur during the transfer of  $\text{CO}_2$  from the vessel to the manometric chamber.

#### ALTERNATE PROCEDURE WITH USE OF CARBON COMBUSTION APPARATUS FOR NINHYDRIN- $\text{CO}_2$ METHOD

This is the original technique of Van Slyke and Dillon (24). One can do only about half as many determinations per hour as with the procedure described in the preceding pages; and as the atmospheric air in the reaction vessel is not removed, the results are less precise, because affected by variations in atmospheric  $\text{CO}_2$ . However, if the carbon apparatus is at hand, and only an occasional isolated ninhydrin- $\text{CO}_2$  analysis is required, with micro or macro (not submicro (see Table I)) amounts, it may be convenient to employ this procedure.

The apparatus is that used by Van Slyke and Folch ((25) Fig. 1), except that the small cylinder marked *F* in the above figure is not needed, although it does no harm if present. No water bath is required. The combustion tube is used as reaction vessel.

The reagents are as described in this paper for the routine ninhydrin method.

In the analysis not more than 2 cc. of amino acid solution is placed in the combustion tube. The amount of ninhydrin used is 50 mg. for 1 cc. of solution, 100 mg. for 2 cc. The first steps of the procedure are the same as are described in the preceding pages of this paper through the stage marked "Chilling the solution."

From this point the procedure is as follows:

2 cc. of 0.5 N NaOH are measured into the manometric chamber, as in the combustion method. The ninhydrin is measured into the combustion tube. The ground joint of the combustion tube is lubricated with a ring of glycerol, and the apparatus is assembled as shown in Fig. 2 of Van Slyke and Folch (25), but with cock *a* open. The solution in the tube is heated to boiling and is boiled gently for 3 minutes when the pH is 4.7, 4 minutes when it is 2.5, 6 minutes when it is 1. During the boiling the leveling bulb is set at such a level that the mercury surface in it is 10 or 20 mm. below the mercury surface in the chamber, so that the boiling is at almost atmospheric pressure. Boiling must be so gentle that the upper half of the glass connecting tube remains cool.

At the end of the boiling period the micro burner is lowered

several cm. below the bottom of the reaction tube, and the gas is passed back and forth between the tube and the chamber six times. At each lowering of the mercury in the chamber the solution boils vigorously, but the heat should be so gentle that no liquid passes over into the chamber.

After six passages the burner is removed from below the tube, the leveling bulb is held at such a level that about 15 cc. of gas are in the chamber, and the cock between the chamber and the reaction tube is closed. The 15 cc. of gas thus trapped in the chamber are freed of  $\text{CO}_2$  by raising and lowering the mercury six times. The unabsorbed gas in the chamber is then ejected.

To transfer the last portions of  $\text{CO}_2$  to the alkali in the chamber, the mercury in the latter is lowered to the middle of the chamber and the chamber is reconnected with the reaction vessel. The latter is gently heated again, and the remaining  $\text{CO}_2$  is absorbed by six more passages of the gas.

The reaction tube is then disconnected from the chamber, and the  $\text{CO}_2$  absorbed in the alkali is determined as described on pp. 525-527 of Van Slyke and Folch (25).

### Calculations

The pressure,  $P_{\text{CO}_2}$  of  $\text{CO}_2$  from amino acid carboxyl groups, is calculated as

$$P_{\text{CO}_2} = p_1 - p_2 - c$$

The correction  $c$  is the value of  $p_1 - p_2$  obtained in the blank analysis.

The carboxyl carbon or carboxyl nitrogen is calculated as

$$\text{Mg. carboxyl carbon or nitrogen} = P_{\text{CO}_2} \times \text{factor}$$

The values of the factors are given in Table II.

The carbon factors are computed by Equation 1 of Van Slyke and Folch (25), based on Van Slyke and Sendroy (28), with the following values for the constants.

	<i>a</i>	<i>i</i>	<i>S</i>
Macroanalysis.....	10	1.007	3
Microanalysis.....	2	1.017	3
Submicroanalysis.....	0.5	1.037	3

*A* in all three analyses is 50; the  $\alpha'$  of the acid lactate solution was determined by the method of Van Slyke (23) and found to be 0.891 times the  $\alpha'$

TABLE II

*Factors by Which  $PCO_2$  is Multiplied to Obtain Mg. of Carboxyl Carbon or Carboxyl Nitrogen*

Temperature	Carboxyl carbon			Carboxyl nitrogen*		
	$\dagger a = 10$ $i = 1.007$	$a = 2$ $i = 1.017$	$a = 0.5$ $i = 1.037$	$a = 10$ $i = 1.007$	$a = 2$ $i = 1.017$	$a = 0.5$ $i = 1.037$
°C.						
15	0.007168	0.001447	0.0003688	0.008361	0.001688	0.0004303
16	133	39	69	320	79	0.0004280
17	097	32	50	278	71	58
18	061	25	32	237	62	37
19	026	18	14	196	54	16
20	0.006991	11	0.0003596	156	46	0.0004195
21	958	04	78	117	38	74
22	926	0.001397	61	078	30	54
23	893	91	44	040	23	35
24	860	84	28	003	15	16
25	827	78	12	0.007964	08	0.0004097
26	797	71	0.0003496	928	0.001599	79
27	767	65	80	893	92	60
28	736	59	65	858	85	42
29	706	53	50	822	78	25
30	676	47	35	787	71	07
31	648	42	20	755	66	0.0003990
32	621	36	06	723	59	74
33	593	30	0.0003392	690	52	57
34	566	25	78	658	46	41
35	538	20	64	626	40	25

\* "Carboxyl nitrogen" = carboxyl carbon  $\times$  14.01/12.01.

$\dagger a$  is the volume in cc. at which  $PCO_2$  is measured, and  $i$  the correction factor for reabsorption of  $CO_2$  (26). The volume ( $S$ ) of solution extracted in the Van Slyke-Neill chamber is 3 cc. for all three types of analyses.

For the most precise results, the factors must, for each apparatus and type of analysis, be multiplied, either by the ratio

Actual  $a$  found by calibration

$a$  assumed in calculation of Table II

or by the empirical  $b$  correction factor found in analyses of a pure amino acid (see text for  $b$  correction factor and Table III for examples of its determination).

of water. Because this value of  $\alpha'$  is somewhat higher than the  $\alpha'$  for the lactate-hydrazine solutions used for carbon combustions (25), the carbon factors for  $a = 2$  and  $a = 10$  in Table II are slightly higher than those applied to carbon combustions (25).

#### *Correction of Factors by Analyses of Pure Amino Acids*

In each apparatus the precise factors for calculation of carboxyl values will as a rule deviate slightly from the factors in Table II, because the volumes of  $a$  and  $A$  in the manometric chamber are likely to deviate slightly from the values assumed for them in the calculation of Table II, the thermometer in the water jacket of the apparatus may have a slight correction, and, if a micro balance is used, its rider may also have its correction. The simplest way to correct for deviations in all these constants is, as in the carbon combustion method (p. 530 (25)), to perform a series of analyses on a pure substance. One of the amino acids is used which gives theoretical results under all conditions of the analysis. All the corrections for the apparatus are included in one factor,  $b$ , which is calculated as

$$b = \frac{\text{theoretical carboxyl N}}{\text{carboxyl N calculated from } P_{\text{CO}_2} \text{ by factor in Table II}}$$

The corrected factors are calculated at  $3^\circ$  intervals as  $b$  times the factors in Table II, and are plotted against temperature for use with the apparatus.

Table III gives an example of data used to determine the correction factors for an apparatus. These data also indicate the order of the constancy of results obtainable by the method.

#### *CO<sub>2</sub> Yields from Different Amino Acids*

The yields of carboxyl CO<sub>2</sub> from most of the amino acids yielded by protein hydrolysis, and also from three others, are shown in Table IV, with variations of the reaction medium.

The reactions in glacial acetic acid were carried out by heating over a free flame in a combustion tube as described on p. 647. The procedure, of preliminary evacuation followed by immersion in a hot bath, for routine analyses in aqueous solution, cannot be applied when the medium is glacial acetic acid, because the reaction starts so quickly that some CO<sub>2</sub> would be lost during the preliminary evacuation. The results with aqueous solutions were

TABLE III

*Analyses of Alanine to Determine Apparatus Correction Factor, b, for Macro-, Micro-, and Submicroanalyses*

Theoretical carboxyl nitrogen = 15.722 per cent.

Type of analysis (see Table I), conditions of reaction, and $\alpha$ volume*	Reaction vessel used (Fig. 1)	Alanine sample	$P_{CO_2}$	Temperature	Carboxyl N factor from Table II, uncorrected	Carboxyl N calculated by uncorrected factor	Correction factor b
		mg.	mm.	°C.		per cent	
Macro	A	14.730†	290.4	24.5	0.007984	15.74	
3 cc. solution	"	17.725†	348.4	24.4	7988	71	
150 mg. ninhydrin	"	17.684†	347.1	24.4	7989	68	
100 " buffer, pH 2.5	"	16.317†	319.3	23.2	8033	72	
$\alpha = 10$ cc. uncorrected,	"	16.650†	325.2	22.5	8059	74	
10.008 cc. by calibration							
		Average . . . . .				15.72	1.000
		Mean deviation from average . . .				$\pm 0.02$	$\pm 0.001$
		Correction factor calculated from calibrated $\alpha$ of 10.008 . . . . .					1.001
Micro	A	3.284†	321.3	25.0	0.001608	15.73	
2 cc. solution	"	3.397†	330.7	25.1	607	65	
100 mg. ninhydrin	"	4.088	403.1	28.0	585	63	
50 " buffer, pH 2.5	"	4.088	403.2	28.0	585	63	
$\alpha = 2$ cc. uncorrected,	B	4.605†	449.5	25.3	605	67	
2.006 cc. by calibration	"	3.340†	327.8	25.4	604	65	
	C	4.088	404.2	28.0	585	67	
	"	4.088	403.4	28.0	585	64	
		Average . . . . .				15.66	1.004
		Mean deviation from average . . .				$\pm 0.03$	$\pm 0.002$
		Correction factor calculated from calibrated $\alpha$ of 2.006 . . . . .					1.003
Submicro	A	0.6771	268.5	33.2	0.0003954	15.68	
1 cc. solution	"	6771	266.6	33.1	55	57	
50 mg. ninhydrin	"	6771	266.2	33.0	57	56	
50 " buffer, pH 2.5	"	6771	267.7	32.7	62	67	
$\alpha = 0.5$ cc. uncorrected,	"	6771	266.2	32.4	67	60	
0.501 cc. by calibration							
		Average . . . . .				15.62	1.006
		Mean deviation from average . . .				$\pm 0.05$	$\pm 0.003$
		Correction factor calculated from calibrated $\alpha$ of 0.501 . . . . .					1.002

\*  $\alpha$  = gas volume at which  $P_{CO_2}$  was measured.

† Samples weighed on micro balance. Other samples were measured as aliquots, within ca.  $\pm 0.001$  cc., of standard solutions.

TABLE IV

*Quantitative Evolution of CO<sub>2</sub> from Amino Acids Heated with Ninhydrin under Varying Conditions of pH, Time, and Solvent*

Concentration of ninhydrin, 50 mg. per cc. in acetic acid and in water at pH 4.7 and 2.5, 42 mg. per cc. in water at pH 1.

Yields reported by the integers 1 or 2 are quantitative and not increased by longer heating; yields reported to two decimal places are increased by longer heating.

Amino acid	Moles CO <sub>2</sub> evolved per mole amino acid				
	In water solution*				In glacial acetic acid† 4 min.
	pH 4.7 6 min.	pH 2.5 7 min.	pH 1‡		
			8 min.	15 min.	
Amino acids found in protein hydrolysates					
Glycine.....	1	0.95	0.80	0.93	1
Alanine.....	1	1	0.97	1	1
Valine.....	1	1		1	1
Leucine.....	1	1		1	1
Serine.....	1	1		1	1
Threonine.....	1	1		1	1
Hydroxyglutamic acid.....	1.03	1		1	
Glutamic acid.....	1.02	1		1	1.14
Aspartic acid.....	2	2		2	1.90
Phenylalanine.....	1	1		1	1
Tyrosine.....	1	1		1	1
Tryptophane.....	1	0.90		0.90	0.56
Proline.....	1	1		1	1
Hydroxyproline.....	1	1		1	1
Cystine.....	1.50	1.89	2.00	2.07	1.28
Methionine.....	1	1		1	
Arginine.....	1	1	1	1	1
Histidine.....	1	1	1	1	0.88
Lysine.....	1.34	1.05	1.00	1.02	1
Hydroxylysine.....	1.07	1.02	1.00	1.01	
Amino acids not usually found in protein hydrolysates					
Homocystine.....	1	1			
Ornithine.....	1.06	1.01	1	1.01	
β-Alanine.....	0.16	0	0	0	0.87

\* Heated by immersion of evacuated reaction vessels in boiling water as described on p. 642 *et seq.*

† Glacial acetic acid solutions were heated with open flame in combustion tubes, as described on p. 647 *et seq.*

‡ The solutions marked "pH 1" contained H<sub>3</sub>PO<sub>4</sub> in 1 M concentration.

obtained by the routine technique, with evacuated reaction vessels. (For the synthetic hydroxyglutamic acid we thank Dr. H. D. Dakin; for the homocystine, cystine, and methionine, Dr. Vincent du Vigneaud.)

In each instance where the yield of  $\text{CO}_2$  is shown by the integer 1 or 2, the yield was theoretical and no additional  $\text{CO}_2$  was evolved when the heating was prolonged beyond the time given. For the exceptional amino acids which do not give precise stoichiometric and completed reactions in the standard reaction intervals, results are given to two decimal places.

The sharp difference in the behavior of the extra carboxyl group of *aspartic acid* from the extra carboxyl groups of the 5-carbon dicarboxylic acids, glutamic and hydroxyglutamic acids, is noteworthy; aspartic acid evolves 2 moles of  $\text{CO}_2$ , while glutamic and hydroxyglutamic evolve only 1  $\text{CO}_2$  each at pH 1 or 2.5, and only 1.02 to 1.03 at pH 4.7.

*Reaction Rates of the Diamino Acids and Alanine at pH 1*  
(1 M  $\text{H}_3\text{PO}_4$ )

Solutions of approximately 0.02 M concentration were prepared of lysine dihydrochloride, histidine dihydrochloride, arginine monochloride, and  $\alpha$ -alanine, and 0.01 M cystine. 2 cc. portions of the solutions were measured with an accurately calibrated pipette into reaction tubes (Fig. 1, A), and were acidified with 0.4 cc. portions of 6 M  $\text{H}_3\text{PO}_4$ . 100 mg. of ninhydrin were added to each and the tubes were evacuated and immersed in boiling water for varying intervals. They were then cooled under the tap and the  $\text{CO}_2$  was determined as described for routine analyses.

The results are given in Fig. 4, which shows that in analyses of histidine, arginine, cystine, and lysine 1 mole of  $\text{CO}_2$  is evolved from each if the boiling period is set at 8 to 9 minutes, in the presence of concentrations of 42 mg. of ninhydrin per cc. and of 1 M  $\text{H}_3\text{PO}_4$ . Arginine and histidine yield 1 mole of  $\text{CO}_2$  in any period longer than 6 minutes, but lysine and cystine react more slowly with their carboxyls, and then slowly evolve additional  $\text{CO}_2$ , so that their time must be empirically set at between 8 and 9 minutes to give theoretical results. If monoamino acids are also present, as exemplified by alanine, one may run the reaction 15 minutes to obtain complete yields from them, and accept a +2



per cent error in the  $\text{CO}_2$  from the lysine and +5 per cent for the cystine.

*Choice of pH for Reaction with Ninhydrin*

From Table IV and Fig. 4 it appears that if one wishes to determine the free amino acids in a mixture, such as a protein hy-

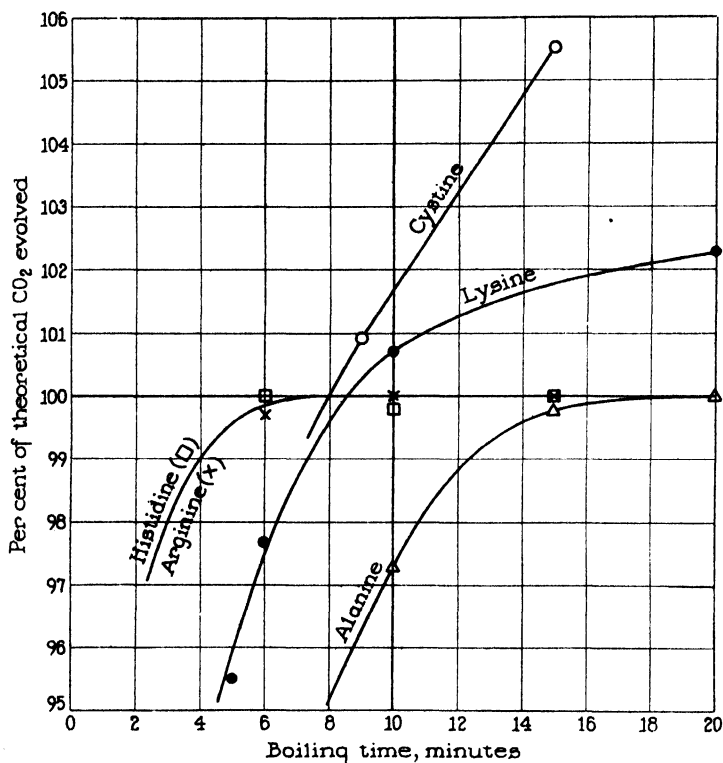


FIG. 4. Time curves of  $\text{CO}_2$  evolution from lysine, arginine, histidine, and alanine reacting at pH 1 with ninhydrin concentration of 42 mg. per cc.

drollysate, blood filtrate, or other biological fluid where probably all the amino acids yielded by protein hydrolysis may be present, results approximating theoretical will be obtained by carrying out the ninhydrin reaction at pH 2.5. There the  $\text{CO}_2$  yields are theoretical with most of the amino acids, the exceptions being glycine, tryptophane, and cystine, which are low (95, 90, and 95 per cent, respectively), and lysine, which is high (105 per cent).

If, however, one separates by precipitation with phosphotungstic acid the mixture of amino acids in a protein hydrolysate, the precipitated group, *viz.* histidine, arginine, lysine, hydroxylysine, and cystine, will yield theoretical results by reaction with ninhydrin at pH 1 and 8 to 9 minutes boiling (with 42 mg. of ninhydrin per cc. of reacting solution), while the "monoamino" group in the filtrate will yield theoretical results at pH 4.7, the only exceptions being the excess of 2 to 3 per cent yielded by glutamic and hydroxyglutamic acids.

TABLE V  
*Peptides and Amino Acid Derivatives*

Substance	Moles CO <sub>2</sub> liberated per mole substance when heated in 5 per cent aqueous ninhydrin solution for 6 min. at pH 2.5	Per cent total N determined as carboxyl N
<b>Peptides</b>		
Glycylproline.....	0.01	0.5
Glycylalanine.....	0.00	0
Glycyltrileucine.....	0.00	0
Glycylalanylglycine.....	0.00	0
Glycylglycine.....	0.016	0.8
Glycyl- <i>l</i> -leucine.....	0.00	0
Glycyl- <i>l</i> -phenylalanine.....	0.00	0
Glutamyl- <i>l</i> -tyrosine.....	0.00	0
<i>l</i> -Leucylglycine.....	0.00	0
Dialanylglycine.....	0.02	0.7
Dileucyldiglycine.....	0.02	0.7
Glutathione.....	1.00	33.3
<b>Other amino acid derivatives</b>		
Glycine anhydride.....	0	0
Glycylamide.....	0	0
N-Dimethylglycine.....	0	0
Hippuric acid.....	0	0
$\alpha$ -Benzenesulfonyllysine.....	0	0
Sarcosine.....	0.8	80
$\epsilon$ -Carbobenzoxyllysine.....	1	50

*Peptides and Other Amino Acid Derivatives of Definite Composition*

The failure of peptides other than glutathione (which has the —CH(NH<sub>2</sub>)·COOH group free) and of certain other amino acid derivatives to evolve CO<sub>2</sub> when boiled with ninhydrin is shown in Table V.

If the peptides which evolve 0.01 or 0.02 mole of  $\text{CO}_2$  in 6 minutes are heated for long periods with ninhydrin, the  $\text{CO}_2$  evolved increases as a linear function of the time. The increase is due presumably to gradual hydrolysis to amino acids.

### *Proteins and Peptones*

Table VI shows that proteins and Witte's peptone yield amounts of  $\text{CO}_2$  so slight as to be ordinarily negligible; the  $\text{CO}_2$  from each of the three proteins indicates carboxyl nitrogen equal to only 0.03 per cent of the protein nitrogen. This slight evolution of  $\text{CO}_2$  appears attributable to hydrolysis of protein in the solution at  $100^\circ$  and pH 2.5. If the reaction vessels are left at  $100^\circ$  for an hour, evolution of  $\text{CO}_2$  continues at the same slow rate observed in the first 6 minutes.

TABLE VI

*Carboxyl Nitrogen Yielded by Peptones and Proteins at pH 2.5*

Ninhydrin concentration 50 mg. per cc.

Substance	Carboxyl N determined in 6 min.
	per cent of total N
Witte's peptone.....	0.21
Serum albumin.....	0.03
Hemoglobin.....	0.03
Casein.....	0.03

When free amino acids are determined in the presence of proteins, the effect of the latter will be negligible, unless the ratio of protein to amino acids is great; protein 100 times the amino acids would cause only a plus error of 3 per cent in the determined free amino acids. Also, the linear time reaction of the proteins makes it easy to correct for the protein effect when it is significant. The correction is made by measuring the "after reaction" as follows:

*Correction for Reaction of Proteins with Ninhydrin*—After the usual ninhydrin- $\text{CO}_2$  determination is finished, the reaction vessel, detached from the chamber *without releasing its vacuum*, is heated again exactly as in the routine analysis, and the  $\text{CO}_2$  formed during the second period is determined. Because of the linear time reaction of the proteins, the  $\text{CO}_2$  formed from them during the second heating period will equal that formed from

them during the first period. Hence the correction for  $\text{CO}_2$  evolved from protein is made by subtracting the  $\text{CO}_2$  evolved during the second heating period (the "after reaction") from that formed during the first period. The difference is the  $\text{CO}_2$  from the free amino acids.

This correction can be used in determining the free amino acids, without removal of proteins, in blood plasma, where the ratio of proteins to free amino acids is of the order of 200.

As shown by Table V, some peptides hydrolyze to the extent of 1 or 2 per cent during the reaction with ninhydrin at pH 2.5. If any such peptides are present in the protein-amino acid mixture, correction for their effect on the free amino acid value is included by the above procedure.

TABLE VII  
*Substances Other Than Amino Acids and Their Derivatives*

Substance	Moles $\text{CO}_2$ liberated per mole substance when heated 6 min. in water solution with 50 mg. of ninhydrin per cc.	
	In water solution at pH 2.5	In glacial acetic acid
Ascorbic acid.....	0.10	
Creatinine.....	0	0.33
Ammonia.....	0	
Glucosamine.....	0	
Ethanolamine.....	0	
Urea.....	0.005	
Creatine.....	0	
Guanidine acetate.....	0	0
Aniline.....	0	0
Dipropylamine.....	0	
Lactic acid.....	0	
Citric ".....	0	
Acetic ".....	0	
Oleic ".....	0	

*Substances Other Than Amino Acids and Their Derivatives*

The only non-nitrogenous organic acid thus far encountered which evolves  $\text{CO}_2$  as the result of reaction with ninhydrin under the conditions of the analysis at pH 2.5 is ascorbic acid (Table VII). It is interesting that *creatinine*, which reacts with ninhydrin in aqueous solution but slightly at pH 4.7 and not at all at pH 2.5, evolved 0.33 mole of  $\text{CO}_2$  when boiled with ninhydrin in glacial acetic acid for 4 minutes. *Ethanolamine* and *glucosamine*, inert at pH 2.5, as shown in Table VII, evolve traces of  $\text{CO}_2$  at pH 4.7. The slight  $\text{CO}_2$  evolution from *urea* is not due to reaction with

ninhydrin, but to hydrolysis by the hot water. Actually only about half as much  $\text{CO}_2$  is evolved in the presence of ninhydrin as in its absence; a combination appears to be formed between urea and ninhydrin which evolves  $\text{CO}_2$  more slowly than does urea.

#### APPLICATIONS OF THE NINHYDRIN- $\text{CO}_2$ METHOD

##### *Analytical Determination of Purity of Amino Acids*

As indicated by Table IV, theoretical results can be obtained with any of the known amino acids yielded by protein hydrolysis if the reaction is carried out at a properly chosen pH. A sample of 4 to 6 mg. suffices for an accuracy of 2 or 3 parts per 1000. The procedure affords one of the quickest and most accurate micro-methods for determination of analytical purity of amino acid preparations.

##### *Determination of Free Amino Acids in Biological Material*

Because of the specificity of the ninhydrin- $\text{CO}_2$  reaction for free amino acids, it is adapted to the determination of free amino acids in complex biological mixtures. As mentioned above, it can be used to determine free amino acids in blood plasma without removal of the proteins. Methods for blood and urine will be published in a later paper.

##### *Estimation of Lysine Plus Hydroxylysine in Diamino Acid Mixtures from Difference between Amino Nitrogen and Carboxyl Nitrogen*

When in a mixture of the diamino acids, lysine, arginine, and histidine, the amino nitrogen is determined by the nitrous acid method (21) and the "carboxyl nitrogen" by reaction with ninhydrin at pH 1, each of the diamino acids has a ratio of 1:1 for  $\text{NH}_2:\text{COOH}$  except lysine, which has a ratio of 2:1. In a mixture of these amino acids therefore, as shown experimentally by Table VIII, the lysine can be calculated from the excess of  $\text{NH}_2$  above  $\text{COOH}$  by the formula

$$\text{Lysine N} = 2(\text{amino N} - \text{carboxyl N})$$

If hydroxylysine is present, it will be estimated with the lysine.

If cystine also is present, as it frequently is when the hexone base fraction of a protein hydrolysate is precipitated with phosphotungstic acid (22), the cystine will not interfere with the lysine

plus hydroxylysine estimation, since cystine, like histidine and arginine, has a 1:1 ratio for  $\text{NH}_2:\text{COOH}$ . In the presence of cystine it will, however, be necessary to add KI to the nitrite of the amino nitrogen reagents, as shown by Kendrick and Hanke (10), to prevent cystine from giving higher than theoretical  $\text{NH}_2$  values.

In the experiment of Table VIII solutions of 10 cc. volume were prepared with varying amounts of lysine, histidine, and arginine. The magnitudes of the carboxyl and amino nitrogen concentrations were similar to those encountered in the diamino acid solutions obtained in the nitrogen distribution method (22) for analysis of hydrolyzed proteins.

Carboxyl determinations were done in duplicate on 1 cc. portions, with measurement of  $\text{CO}_2$  pressures with the gas at 2 cc. volume. Since the experiments were done before the present technique with preliminary evacuation had been introduced, the original procedure of Van Slyke and Dillon (24) was followed. The reaction with ninhydrin was carried out at pH 2.5, with exactly 3 minutes boiling over a free flame, the time being counted from the moment when boiling began. These conditions had been found to give theoretical carboxyl values for lysine as well as the other diamino acids; if, however, the boiling were continued longer than 3 minutes, the values found for lysine would be too high. The present procedure, with preliminary evacuation and a pH of 1, demands less care and skill to yield theoretical carboxyl values with the hexone base mixture, but the data in Table VIII show that even the earlier technique was adequate.

For amino nitrogen determination by the nitrous acid reaction, 3 cc. portions of each solution were diluted to 30 cc., and 5 cc. aliquots, representing 0.5 cc. of the original base solution, were used for duplicate analyses by the manometric method (21). 20 minutes were allowed for the reaction with nitrous acid at 21–23° to become complete with the  $\omega\text{-NH}_2$  of the lysine.

The results are given in Table VIII. The maximum error of 0.014 mg. of lysine nitrogen per cc. in a protein analysis by the nitrogen distribution method (22) would be of the order of 0.2 per cent of the total protein nitrogen.

#### *Estimation of Aspartic Acid in Amino Acid Mixtures*

The ability of aspartic acid to evolve 2 moles of  $\text{CO}_2$  in the reaction with ninhydrin makes it possible by this reaction to determine aspartic acid in mixtures containing most of the other amino acids yielded by protein hydrolysis. As a simple case, in the mixture of glutamic and aspartic acids obtained from protein hydrolysates by the barium method of Jones and Moeller (9),

the proportion of aspartic acid can be determined by subtracting from the carboxyl nitrogen determined at pH 2.5 the amino nitrogen determined by the nitrous acid method (20).

$$(4) \quad (\text{Aspartic acid N}) = (\text{COOH-N}) - (\text{NH}_2\text{-N})$$

Similarly aspartic acid can be measured by the excess of carboxyl nitrogen over amino nitrogen in mixtures with such amino acids

TABLE VIII

*Analysis of Known Hexone Base Mixtures with Calculation of Lysine from Difference between Amino N and Carboxyl N*

Mixture.....	A	B	C	D	E
	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.
Added substances					
Arginine dinitrate..... <chem>C6H14N4O2.2HNO3</chem>	0	1.908	2.543	1.387	0.763
Histidine dichloride..... <chem>C6H8N2O2.2HCl</chem>	10.20	7.012	4.675	0	3.927
Lysine dichloride..... <chem>C6H14N2O2.2HCl</chem>	1.002	2.755	4.592	10.018	6.612
Found					
Amino N.....	0.750	0.868	0.991	1.340	1.115
Carboxyl N.....	0.693	0.699	0.697	0.700	0.694
Lysine N calculated as 2(amino N-carboxyl N).....	0.114	0.338	0.588	1.280	0.842
Calculated from added substances					
Amino N.....	0.754	0.872	0.993	1.345	1.122
Carboxyl N.....	0.690	0.696	0.699	0.705	0.699
Added lysine N.....	0.128	0.352	0.587	1.280	0.845
Difference between lysine N calculated from analyses and lysine N added.....	-0.014	-0.014	+0.001	0	-0.003

as alanine, valine, etc., in which carboxyl and amino nitrogen are equal. Cystine, like aspartic acid, yields 2 moles of  $\text{CO}_2$ , but the ratio of amino nitrogen to carboxyl nitrogen is 1:1, as in the monoaminomonocarboxylic acids.

Lysine, hydroxylysine, proline, and hydroxyproline are the only known amino acids yielded by protein hydrolysis in the presence of which aspartic acid could not be measured by the above formula from carboxyl values determined at properly chosen pH.

In the presence of proline and hydroxyproline, however, aspartic acid could be calculated by Equation 5.

$$(5) \quad (\text{Aspartic acid N}) = (\text{COOH-N}) - (\text{total N})$$

In the monoamino acid fraction obtained after removal of the hexone bases from protein hydrolysates, only tryptophane with its 2 nitrogen atoms would apparently interfere with the calculation of aspartic acid by Equation 5, if the ninhydrin reaction is carried out at pH 4.7; and a correction could be made for the extra nitrogen of the tryptophane after colorimetric estimation of this amino acid. Application of Equation 5 to the monoamino fractions of protein hydrolysates (unpublished) has in fact yielded results which agree fairly well with the amounts of aspartic acid expected from other data in the literature.

#### *Determination of Free Amino Acids in Protein Digests*

As shown in Tables V and VI, proteins react with ninhydrin so slightly that only 0.03 per cent of their nitrogen is determined as "carboxyl nitrogen," and peptides, except those of the glutathione structure, react either not at all or only in traces. It appears therefore that the ninhydrin-CO<sub>2</sub> method offers a means for determining with but little error the free amino acids in the mixtures of peptides and proteins encountered in protein digests.

To obtain an indication of the pH most suited for the ninhydrin reaction in protein digests aliquots of a casein digest were heated for varying periods, with ninhydrin in 5 per cent concentration, at pH 1, 2.5, and 4.7, and time curves of the CO<sub>2</sub> production at these pH levels were plotted. The digest had approximately two-thirds of its amino acids completely liberated, while the other third was still in the form of peptides.

The results are shown in Fig. 5. It is seen that the curves of CO<sub>2</sub> production at pH 1 and 2.5 rise sharply to the same maximum, which is reached in the same time required for free amino acids to complete the reaction (Fig. 2). On the other hand the reaction at pH 4.7 does not reach a definite maximum in this time, but continues slowly to rise to higher levels, indicating formation of additional CO<sub>2</sub>, presumably from secondary reactions of some of the aldehydes formed by the primary reaction. It appears



therefore that pH 2.5 or 1 is preferable to pH 4.7 for the reaction in protein digests.

The presumable sources of the slow secondary formation of CO<sub>2</sub> at pH 4.7 are lysine, glutamic acid, and hydroxyglutamic acid. As is seen in Table IV, these all yield more than theoretical amounts of CO<sub>2</sub> in the regular reaction period at pH 4.7, but not

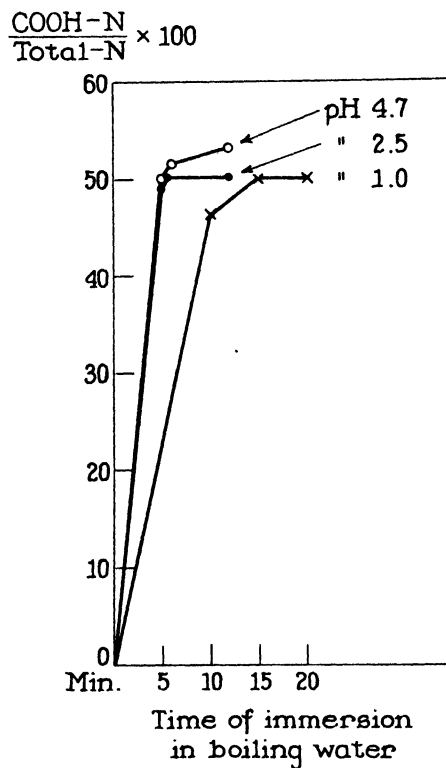


FIG. 5. Time curves of CO<sub>2</sub> formation of casein digest heated with ninhydrin at 50 mg. per cc. concentration and at pH 1, 2.5, and 4.7.

at pH 2.5, except for a diminished amount from lysine. With prolongation of the reaction the excess formation of CO<sub>2</sub> increases. The amounts of these three amino acids in casein estimated in Schmidt's summary (18) total 38 gm. per 100 gm. of casein; these amounts would about account for the extent, shown in Fig. 4, by which the CO<sub>2</sub> yielded at pH 4.7 exceeded that at pH 2.5 and 1.

*Experiment Illustrating Liberation of Peptides and Free Amino Acids  
in Tryptic Digestion of Casein and Demonstrating Absence  
of Peptidase from Crystalline Trypsin*

Two preparations of trypsin were used. One was a crystalline trypsin prepared according to Northrop and Kunitz (13). The other was Fairchild's commercial trypsin, which obviously contained peptidase as well.<sup>12</sup>

150 mg. of casein were dissolved in about 15 cc. of water plus 18 to 20 mg. of  $\text{Na}_2\text{CO}_3$ . The solution was brought to pH 8 by addition of 0.05 N HCl, 15 mg. of trypsin were added, and the solution was diluted to 20 cc. The mixtures were digested at 37°. At intervals samples of 1 cc. were withdrawn and analyzed for amino nitrogen by the nitrous acid method (21) and for free amino acids by the ninhydrin- $\text{CO}_2$  reaction at pH 2.5. The initial values were estimated from separate analyses of solutions of casein and of trypsin. The total nitrogen was determined by micro-Kjeldahl. The results are shown in the curves of Fig. 6.

Striking is the absence of free amino acids in the digest formed with *crystallized trypsin*. When digestion had reached its limit, less than 1 per cent of the total nitrogen was in the form of free amino acids, as shown by the ninhydrin- $\text{CO}_2$  method. The increase in amino nitrogen, by the nitrous acid method, showed that digestion progressed as far as formation of peptides, but the carboxyl determination showed that practically no free amino acids were liberated. Our negative results for peptidase activity in the crystalline trypsin agree with negative results of Northrop and Kunitz (13), who observed no measurable hydrolysis of pentaglycylglycine, tri-*l*-alanyl-*l*-alanine, and tetra-*dl*-alanyl-*dl*-alanine.

Comparison of the amino nitrogen, measured by the nitrous acid method, set free by the digestion (8.7 per cent of the total nitrogen) with the amount freed by total hydrolysis (65 per cent of the total nitrogen, as found in other experiments), indicates that the peptides formed when digestion by crystalline trypsin reached its limit contained an average of seven or eight amino acids per peptide molecule.

In contrast to the crystalline trypsin the *crude trypsin* evidently contained peptidase in addition to the proteinase. The 15 per cent of nitrogen in the form of carboxyl nitrogen, compared with the 75 per cent formed by complete hydrolysis, indicates that about

<sup>12</sup> For the crystalline trypsin we thank Dr. J. S. Fruton.

20 per cent of the amino acids in the protein was liberated. The rest of this digest was in peptides of about the average size of tetrapeptides, as calculated from the ratio between the peptide amino

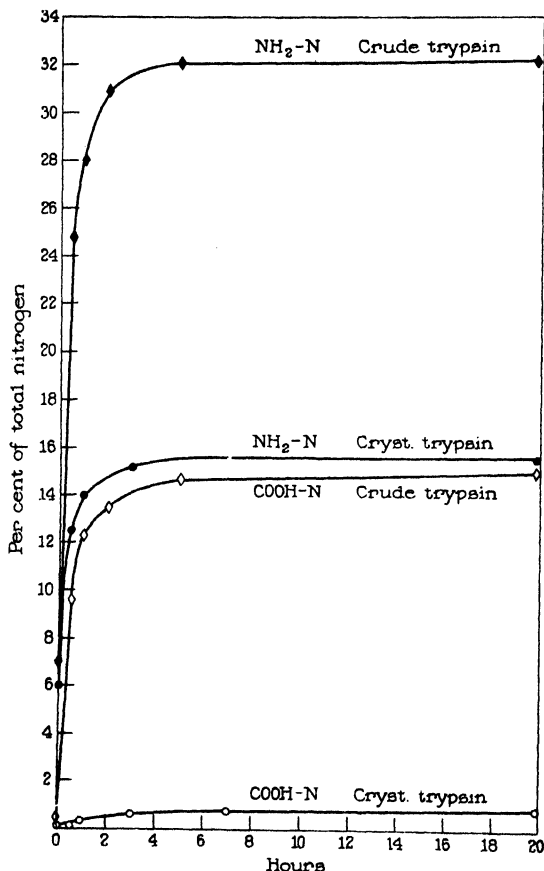


FIG. 6. Digestion of casein with crystalline trypsin and with crude trypsin. Comparison of rise in amino nitrogen determined by the nitrous acid method (free amino acid  $\text{NH}_2$  plus peptide  $\text{NH}_2$ ) with rise in "carboxyl nitrogen" (free amino acids only) during the digestions.

nitrogen of the digest (amino nitrogen freed by the digestion minus carboxyl nitrogen) and the amount of amino nitrogen (65 per cent of the total N) liberated by complete hydrolysis. (This calculation leaves prolyl peptides out of account.)

CO<sub>2</sub> EVOLUTION BY AMINO ACIDS AND PEPTIDES TREATED  
WITH CHLORAMINE-T

The *apparatus* was the same described for the analysis with ninhydrin. The tubes shown in Fig. 1, A were employed for the analyses recorded in Table IX.

The *reagents* were also the same with two exceptions:

In place of the 0.5 N NaOH solution used for absorbing the CO<sub>2</sub> evolved by the ninhydrin reaction, the solution containing 0.5 N NaOH plus 0.3 M hydrazine, employed by Van Slyke and Folch (25) in their carbon method, is used. The chloramine-T gives off enough chlorine to tarnish the mercury in the chamber unless the hydrazine is added to the alkali.

In place of the solid ninhydrin a solution of chloramine-T was used as decarboxylating reagent. The solution was prepared by dissolving the chloramine-T (sodium salt) in 10 parts by weight of water. To this solution was added one-tenth its volume of saturated barium hydroxide solution. After standing half an hour or longer to complete precipitation of the CO<sub>2</sub> which was usually present as traces of carbonate in the chloramine-T, the solution was filtered into a bottle protected from atmospheric CO<sub>2</sub>. The bottle was then connected with a burette, with a blunt outlet tip that could be fitted tightly into the 3 mm. rubber tube of the reaction vessel (Fig. 1). Burette and bottle were arranged so that delivery of 0.5 cc. portions of the solution could be made into the reaction vessel without exposure to atmospheric CO<sub>2</sub>. The alkaline solution thus prepared could be used for a week or longer.

*Procedure*—The amino acid, with 1 cc. of water, was placed in the reaction vessel. 50 mg. of citrate buffer were added for reaction at pH 2.5 or 4.7. For reaction in alkaline solution, no buffer was added; the alkali added as the barium hydroxide in the chloramine-T solution titrated to approximately 0.02 N in the reacting solution.

When the reaction was to be run for only 2 minutes, the vessel and amino acid solution were heated to 45–46° before the chloramine-T solution was added. When the reaction was to be run for 10 minutes, however, the chloramine-T was added without previous warming of the reaction vessel.

After the amino acid solution and buffer had been placed in the reaction vessel, the latter was closed with the adapter and evacu-

ated. The lumen of the rubber tube above the clamp was filled with water, in order to prevent air from entering the vessel with

TABLE IX

*Evolution of CO<sub>2</sub> from Amino Acids, Peptides, Urea, and Creatinine Reacting with Chloramine-T at 45°, at Varying pH and Time. Microanalyses with P<sub>CO<sub>2</sub></sub> Measured at 2 Cc. Volume*

Volume of reacting solution, 1.5 cc.; chloramine-T present, 45 mg.

Amino acid	Moles CO <sub>2</sub> evolved per mole substance				
	pH 2.5		pH 4.7	Alkaline 0.02 N Ba(OH) <sub>2</sub> (no buffer added)	
	2 min.	10 min.	10 min.	2 min.	10 min.
Glycine.....	0.870	0.972	1.255	0.511	0.592
Alanine.....	0.952	1.030	1.017	1.012	1.026
Leucine.....	1.017	1.021	1.022	1.002	1.008
Serine.....	0.925	1.028	1.022		0.993
Threonine.....	0.881	1.015	1.048		0.983
Glutamic acid.....	1.006	1.063	0.996	0.995	1.003
Aspartic acid.....	1.665	1.750	1.938	1.180	1.272
Phenylalanine.....		0.946	1.001	0.998	0.998
Tyrosine.....	1.001	1.033	0.837	0.983	
Tryptophane*.....	0.576	0.751	0.759	0.790	0.982
Proline.....	0.328	0.583	0.995	0.966	1.000
Hydroxyproline.....	0.336	0.805	1.001	0.987	0.995
Cystine†.....	1.067	1.064	0.794	0.642	0.740
Arginine.....	1.013	1.023	1.006	1.003	1.000
Histidine.....	1.018	1.037	1.020	0.976	1.023
Lysine.....	0.988	1.009	1.043	0.995	1.000
Glycylphenylalanine.....			0.075		
Glutamyltyrosine.....			0.069		
Leucylglycine.....			0.005		
Glycylleucine.....			0.028		
Glycylglycine.....			0.032		
Urea.....			0.005	0.0004	0.0003
Creatinine.....			0.002		0.002
β-Alanine.....			0.025		

\* Tryptophane formed with chloramine-T a brown precipitate. None of the other substances analyzed showed this reaction.

† Theoretical for cystine is 2 moles of CO<sub>2</sub>.

the reagent solution next added. The blunt tip of the burette delivering the chloramine-T reagent solution was forced into the

tube as far as the clamp, and the clamp was removed during delivery of 0.5 cc. of the reagent into the vessel. The clamp was restored, and the vessel was disconnected from the burette without admission of any air. The rubber tube above the clamp was washed out with acidified water, in order to prevent absorption of atmospheric  $\text{CO}_2$  by adherent alkaline reagent solution.

The vessel was then placed in a bath at  $45\text{--}46^\circ$  for either 2 or 10 minutes.

At the end of this reaction period, 0.5 cc. of 2 N lactic acid was run into the vessel from a burette in the same manner described for addition of the chloramine-T reagent. The vessel was then attached at once, while warm, to the chamber of the manometric apparatus, which had already been charged with 2 cc. of 0.5 N alkali solution containing hydrazine.

The transfer of the  $\text{CO}_2$  from the vessel to the alkali-hydrazine solution in the chamber and the subsequent steps of the analysis were as described for the analysis with ninhydrin as decarboxylating reagent.

*Calculations*—To calculate carboxyl carbon the carbon factors of Folch and Van Slyke (25) were used, instead of the factors in this paper, since the NaOH-hydrazine solution was used to absorb the  $\text{CO}_2$ . The factors are about 0.5 per cent lower than those in Table II, because the solubility of  $\text{CO}_2$  in the acidified hydrazine-containing solution is less than in the acidified 0.5 N NaOH used for the ninhydrin analysis.

*Results*—The results are given in Table IX. The analyses reported with "alkaline reaction about 0.02 N  $\text{Ba}(\text{OH})_2$ " were done without the addition of any pH-regulating substance other than the barium hydroxide present in the chloramine-T solution. The degree of reproducibility of the results cannot be stated with precision, because repeated analyses were not run with most of the amino acids. The low results with cystine, which should yield 2 moles of  $\text{CO}_2$ , may be due to the insolubility of this amino acid at the temperature used; it did not appear to go completely into solution. The same holds for tyrosine at pH 4.7.

Some of the amino acids yield theoretical results. The reaction of lysine at pH 2.5 or in alkaline solution is more sharply quantitative than when ninhydrin is used as the reagent. In general, however, the results with chloramine-T vary more frequently from theoretical than the results with ninhydrin, and the chloramine-T

reaction shows greater effects of pH variations. Also the peptides are split further with chloramine-T than with ninhydrin.

#### SUMMARY

An analytical method for free amino acids is described in which  $\text{CO}_2$  from their carboxyl groups is evolved in a few minutes by reaction with ninhydrin, and is measured in the Van Slyke-Neill manometric apparatus. The same apparatus and technique serve for micro- and macroanalyses. The precision and rapidity of the method are such that it affords a convenient criterion of the analytical purity of isolated amino acids.

Each of the known amino acids yielded by protein hydrolysis evolves at properly chosen pH 1 mole of  $\text{CO}_2$ , except aspartic acid and cystine, which evolve 2. Glutamic acid, unlike aspartic, evolves  $\text{CO}_2$  from only one carboxyl group.

The  $\text{CO}_2$ -forming reaction is uniquely specific for free amino acids, because it requires the presence, in the free, unconjugated state, of both the carboxyl group, and of the  $\text{NH}_2$  or (in proline and hydroxyproline) the  $\text{NH}(\text{CH}_2)$  group.

Peptides as a class yield no  $\text{CO}_2$ , or only traces, in the analysis. An exception among peptides is glutathione, in which glutamic acid is so linked that the  $-\text{CH}(\text{NH}_2) \cdot \text{COOH}$  group is free.

Chloramine-T as a decarboxylating reagent gives results similar to those of ninhydrin, but less sharply quantitative.

When combined with the nitrous acid method for amino nitrogen, the ninhydrin carboxyl method serves to estimate certain amino acids in mixtures with others. In mixtures of the diamino acids, the excess of  $\text{NH}_2$  over  $\text{COOH}$  serves to measure the lysine plus hydroxylysine. In mixtures of aspartic acid with glutamic acid, alanine, etc., the excess of determinable  $\text{COOH}$  over the  $\text{NH}_2$  or total nitrogen serves as a measure of the aspartic acid.

In protein digests carboxyl determinations indicate the amounts of free amino acids formed.

Crystalline trypsin was thus shown to digest casein to peptides, without liberation of free amino acids.

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# ✓ DETERMINATION OF FREE AMINO ACIDS BY TITRATION OF THE CARBON DIOXIDE FORMED IN THE REACTION WITH NINHYDRIN

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In the present method the  $\text{CO}_2$  evolved by decarboxylating amino acids with ninhydrin is transferred to standard barium hydroxide and titrated. The conditions for making the ninhydrin reaction quantitative are those previously worked out for application in the manometric method (6, 7). The transfer of the  $\text{CO}_2$  to the barium hydroxide is done by a distillation *in vacuo* which is completed in 2 to 3 minutes. The titration, with added barium chloride to insure complete precipitation of the barium carbonate formed, follows a principle used by Krogh and Rehberg (2).

After the present method was completed Christensen, West, and Dimick (1) published a procedure in which Van Slyke and Dillon's (6) conditions for the quantitative ninhydrin reaction were applied, and, as in the present method, the  $\text{CO}_2$  was distilled into standard barium hydroxide and titrated. We nevertheless publish the present method because it is carried out somewhat more rapidly, and with cheaper and simpler apparatus. A pair of small Erlenmeyer flasks attached to a U-tube provides all the special apparatus required for the reaction, the distillation, and the titration.

The titration yields results of the same order of constancy as the manometric method in the macro- and microanalyses. In the submicroanalysis, however, (carboxyl carbon under 0.1 mg.) the titration shows less constancy than the manometric procedure.

When the manometric apparatus is available, the analysis based on it will usually be preferred, particularly for small amounts. The manometric procedure requires fewer precautions against

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atmospheric CO<sub>2</sub>, is more accurate for minimal amounts of amino acids, and is free from the necessity of continual restandardization of solutions. With regard to rapidity there is little to choose between the two methods: each permits about six analyses per hour.

In the following description the terms "macro," "micro," and "submicro" will be used to indicate analyses of amounts of amino acids similar to the amounts used for macro-, micro-, and submicro-analyses in the manometric procedure (7). "Macro" will indicate analyses in which the carboxyl nitrogen measured is from 3 to 5 mg.; "micro," analyses with 0.15 to 1 mg.; and "submicro," analyses with less than 0.15 mg.

The term "carboxyl nitrogen" is used as previously (7) to indicate carboxyl carbon  $\times 14/12$ ; in each amino acid except aspartic acid, "carboxyl nitrogen" is synonymous with  $\alpha$ -amino or (in proline and hydroxyproline) with  $\alpha$ -imino nitrogen.

### *Apparatus*

The apparatus, two Pyrex 25 cc. Erlenmeyer flasks and a U-tube, is shown in Fig. 1. The U-tube and necks of the flasks should be of approximately the same external diameter, 16 or 17 mm. The rubber tubing connecting them is of  $\frac{1}{2}$  inch (12 mm.) bore and  $3/16$  inch wall thickness. The precautions with regard to selection and cleaning of the rubber are the same as those described for the manometric procedure (p. 633 (7)). The 25 cc. flasks should be chosen with but slight flanges, or should have the flanges cut off, or pushed in with a flame. The 25 cc. flasks may if desired be replaced by the special test-tubes used for the manometric analyses (Fig. 1, A of the preceding paper (7)), which are perhaps more convenient for the submicrotitrations.

A 5 cc. burette accurate to 0.01 cc.

A Rehberg (4, 5) micro burette of 200 c.mm. capacity, needed only if submicroanalyses are to be done.

A reservoir for CO<sub>2</sub>-free air. One is conveniently made of two aspirator bottles of 2 liters each, with their lower openings connected by a rubber tube. The bottles are charged with 2.5 liters of 10 per cent NaOH solution. The upper opening of one bottle is closed by a soda lime tube, that of the other by a perforated stopper connected with light rubber tubing ending in a glass

capillary from which  $\text{CO}_2$ -free air can be drawn. When all the air from this bottle has been used, the soda lime tube and air exit tube on the two bottles are interchanged, the bottle now full of solution is elevated, and  $\text{CO}_2$ -free air is drawn from the other, after it has been shaken to assure absorption of all the  $\text{CO}_2$ . The bottles are marked at intervals indicating 200 cc. for convenience in measuring air outflow.

Calibrated glass spoons for convenient measurement of 50 and 100 mg. charges of ninhydrin and citrate buffer. These are the same as previously described (7).

A water bath similar to that used for the manometric method (7). If several analyses are to be run simultaneously, which can easily be done, a rectangular bath will be somewhat preferable to a cylindrical one.

### Reagents

Besides the citrate buffers and ninhydrin, described in the preceding paper (7), the following will be needed.

*Approximately 0.25 N barium hydroxide containing 2 per cent of barium chloride. For macroanalyses.* A saturated solution of barium hydroxide is titrated and diluted to bring its concentration to 0.3 N. 5 volumes of this solution are mixed with 1 volume of a neutral solution of barium chloride containing 12 gm. of  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  per 100 cc.

*Approximately 0.125 N barium hydroxide containing 2 per cent of barium chloride. For microanalyses.* Barium hydroxide is prepared of 0.15 N concentration, and 5 volumes are mixed with 1 volume of the 12 per cent barium chloride.

*Approximately 0.0155 N barium hydroxide containing 10.5 per cent of barium chloride. For submicroanalyses.* 1 volume of the 0.125 N barium hydroxide is diluted with 7 volumes of the 12 per cent neutral barium chloride solution.

*Standard 0.1428, 0.07138, and 0.02855 N HCl (N/7, N/14, and N/35,  $\times 14.00/14.01$ ).* These concentrations are chosen because 1 cc. portions are equivalent to 1, 0.5, and 0.2 mg. of carboxyl nitrogen, which is more frequently calculated than carboxyl carbon.

*Approximately 10 per cent sodium hydroxide for use in the reservoir of  $\text{CO}_2$ -free air.*

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*Caprylic alcohol*, as anti-foam. A good commercial product need not be redistilled for this method.

*Indicators.* 1 per cent phenolphthalein in 95 per cent alcohol. 0.04 per cent cresol red solution in water.

*Sodium veronal buffer of pH 8.0.* This buffer is prepared, according to the directions of Michaelis (3), from a stock solution containing 10.3 gm. of sodium veronal in 500 cc. of water. 7 cc. of stock solution are mixed with 4 cc. of N/14 HCl. The solution is used as color standard for the end-point in the submicrotitration.

### PROCEDURE

#### *Reaction with Ninhydrin*

The amino acid solution is placed in flask *A*, Fig. 1. If the analysis is a submicro one, the solution should not exceed 2 cc. in volume; for micro- or macroanalyses the volume may be as high as 5 cc. As described in the preceding paper (7), buffer is added to give a pH of 2.5 or 4.7, a drop of octyl alcohol is added, preformed CO<sub>2</sub> is boiled off, the flask is stoppered, and the solution is cooled to below 15°.

Flask *B* is now freed of atmospheric CO<sub>2</sub> by passing through it 250 cc. of CO<sub>2</sub>-free air. After the first 100 cc. have been run through, barium hydroxide solution is pipetted into the flask as follows: for the macroanalysis 3 cc. of 0.25 N, for the micro- 1.000 cc. of 0.125 N, and for the submicroanalysis 1.000 cc. of 0.0155 N hydroxide. The stream of CO<sub>2</sub>-free air is continued through the flask while the barium hydroxide is being pipetted in.

Into the amino acid solution in flask *A* one now dumps 50, 100, or 150 mg. of ninhydrin from a glass spoon (see p. 635, preceding paper (7)). Both flasks are quickly connected with the U-tube, as shown in Fig. 1, the lower ends of the rubber connecting tubes being first dipped in water for lubrication. The apparatus is immediately evacuated with a water pump, and the clamp at the top is closed. Several analyses may be prepared as far as this stage, and then boiled together.

The entire apparatus is now immersed upright as far as the clamp in a bath of boiling water for the time necessary to complete the reaction with ninhydrin (see Fig. 2 of the preceding paper (7)). If the concentration of ninhydrin is 50 mg. per cc., this time is 6

minutes at pH 4.7 and 7 minutes at pH 2.5. If the ninhydrin is more dilute, the time is increased in proportion to the dilution.

### *Distillation of CO<sub>2</sub>*

The distillation of the CO<sub>2</sub>, with most of the water, from *A* into *B* is accomplished simply by lifting *B* over the edge of the hot water bath and immersing the lower half of *B* in cold water, while

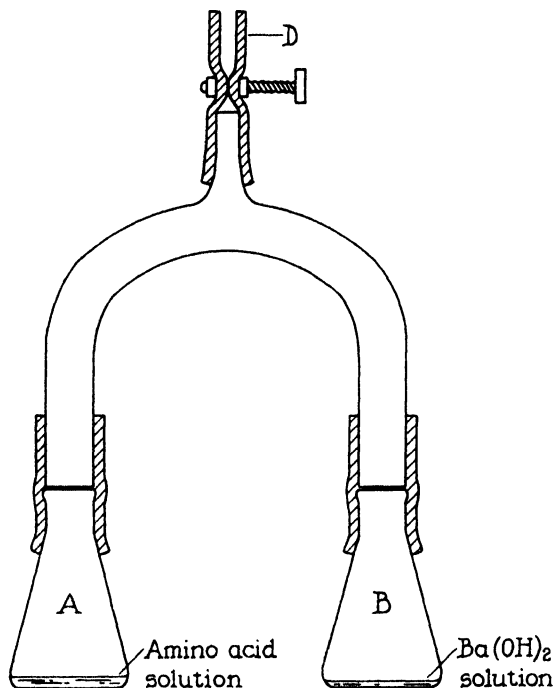


FIG. 1. Apparatus for evolution and distillation of carboxyl CO<sub>2</sub>

*A* and the limb of the U-tube above *A* remain in the boiling water. The time used to complete the distillation of CO<sub>2</sub> into *B* is 2 minutes when the volume of amino acid solution in *A* is 1 or 2 cc.; 3 minutes when it is 3 to 5 cc. The receiving flask is shaken during the distillation to mix the distillate with the barium hydroxide solution. Without the shaking absorption of CO<sub>2</sub> is incomplete.

When the distillation is finished, the apparatus is cooled and

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CO<sub>2</sub>-free air from the reservoir is admitted through *D*. *D* is then closed again with the clamp and the apparatus is left connected until one is ready for the titration.

### *Titration*

*Macroanalyses*—The titration is done with the 0.1428 N (N/7) HCl from a 5 cc. burette. 1 drop of the phenolphthalein solution is added as indicator. No especial precautions against atmospheric CO<sub>2</sub> are required, except that the titration is carried out at once after the flask is disconnected from the U-tube.

*Microanalyses*—The titration is done with the 0.02855 N (N/35) HCl, with the 5 cc. burette and the same technique used in the macroanalyses.

*Submicroanalyses*—The titration is done with 0.07138 N (N/14) HCl and the Rehberg micro burette. Immediately after disconnecting from the U-tube, the flask is placed on the stand of the Rehberg burette and a stream of CO<sub>2</sub>-free air is started bubbling through the barium hydroxide solution as rapidly as it can go without splashing the solution up on the walls of the flask. A drop of cresol red solution is added, and acid from the burette is run in from the submerged tip until the color of the titrated solution matches that of an equal volume of veronal buffer solution containing 1 drop of the cresol red.

### *Blank Analyses*

With each series of micro- or submicrotitrations duplicate blank analyses are done on the same day. The blank analysis is performed with an equal volume of water in place of the amino acid solution, and with all the reagents used in the analysis except the ninhydrin. Ninhydrin evolves no CO<sub>2</sub> from itself, and one can safely economize on the expensive reagent by omitting it from the blanks. The volume,  $T_1$ , of standard HCl required in the blank analysis to neutralize the barium hydroxide after the distillation serves as the basis of the calculation in the analyses of amino acids. It is not necessary to determine the traces of CO<sub>2</sub> yielded by the water and reagents in the blank. The  $T_1$  value includes correction for these, as well as standardization of the barium hydroxide solution. For the macroanalyses the blanks need not be repeated for every series.

*Calculation*

If  $T_1$  represents the cc. of N/7, N/14, or N/35 HCl used in titrating the blank, and  $T_2$  the cc. of the same HCl used in the amino acid analysis, the results are calculated as:

$$\text{Mg. carboxyl nitrogen or carboxyl carbon} = (T_1 - T_2) \times \text{factor}$$

For carboxyl nitrogen the factor is 1, when N/7 HCl is used (macroanalyses); 0.2 when N/35 HCl is used (micro-); 0.5 when the HCl is N/14 (submicro-).

For carboxyl carbon the corresponding factors are 0.857, 0.1715, and 0.4285.

## EXPERIMENTAL

*Effect of Laboratory Air on Titration in Submicroanalyses*

Titration flasks were washed out with 250 cc. of CO<sub>2</sub>-free air while 1 cc. of 0.0155 N barium hydroxide solution was measured into each. Titrations done at once, with CO<sub>2</sub>-free air bubbling through the solutions, showed neutralization of 232.5 and 234.0 c.mm. of N/14 HCl in duplicates.

Control titrations, with all conditions the same except that after the barium hydroxide was measured into the flasks the latter were permitted to stand open for 5 minutes, without passage of a stream of CO<sub>2</sub>-free air, before the titration was begun, took 220.2 and 224.2 c.mm. of N/14 HCl. The difference of 10 c.mm. was equivalent to 0.005 mg. of carboxyl nitrogen, or enough to introduce a 5 per cent error in a determination of 0.1 mg.

Another pair of controls in which there was likewise a delay of 5 minutes, but during this time laboratory air was kept out by passing a stream of CO<sub>2</sub>-free air through the flasks, gave the same results, viz. 232.5 and 232.9 c.mm., as when the titration was done immediately.

*Analyses of Amino Acids*

The conditions under which the different amino acids react quantitatively have already been shown (7). Hence here will be given only data (Tables I to III) obtained with standard alanine solutions to indicate the orders of constancy shown by the three types of titrimetric analyses.



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TABLE I

### Macroanalyses of Alanine

Volume of solution, 3 cc.; ninhydrin, 150 mg.; pH 2.5; boiling time for reaction, 7 minutes; distillation time, 3 minutes.

Theoretical carboxyl nitrogen in all analyses, 3.891 mg.

$T_1 = 5.95$  cc. of 0.1428 N HCl used in blank analysis.

0.1428 N $T_2$ HCl used in titration	$T_1 - T_2$	Carboxyl N	
		Found	Per cent of theory
cc.	cc.	mg.	
2.07	3.88	3.88	99.7
2.09	3.86	3.86	99.4
2.07	3.88	3.88	99.7
2.07	3.88	3.88	99.7
2.09	3.86	3.86	99.4
2.07	3.88	3.88	99.7
Average.....			99.6
Mean deviation from average.....			$\pm 0.1$

TABLE II

### Microanalyses of Alanine

Volume of solution, 1 cc.; ninhydrin, 50 mg.; pH 2.5; boiling time for reaction, 6 minutes; distillation time, 2 minutes.

Sample	$T_1$ Blank titration	$T_2$ Titration in analysis	$T_1 - T_2$ Equivalent of BaCO <sub>3</sub> formed	Carboxyl N found		
mg.	cc. 0.08855 N HCl (N/35)	cc. 0.08855 N HCl	cc. 0.08855 N HCl	mg.	per cent of substance	per cent of theory
3.688*	4.33	1.43	2.90	0.580	15.73	100.1
3.245*	4.33	1.80	2.53	0.506	15.59	99.2
2.777*	4.33	2.16	2.17	0.434	15.63	99.4
1.349	4.33	3.27	1.06	0.212	15.7	100.0
3.604	4.12	1.32	2.80	0.560	15.54	98.9
3.604	4.12	1.30	2.82	0.564	15.65	99.6
3.604	4.12	1.29	2.83	0.566	15.70	99.9
3.604	4.12	1.30	2.82	0.564	15.65	99.6
Average.....					99.6	
Mean deviation from average.....					$\pm 0.3$	

\* Weighed on micro balance. Other analyses were on aliquots of standard solutions.

TABLE III  
*Submicroanalyses of Alanine*

Volume of solution 1 cc.; ninhydrin, 50 mg.; pH 2.5; boiling time for reaction, 6 minutes; distillation time, 2 minutes.

All analyses on 1.000 cc. aliquots of standard solutions.

Reaction vessel	Sample	$T_1$ Blank titration	$T_2$ Titration in analysis	$T_1 - T_2$ : Equivalent of $\text{BaCO}_3$ formed	Carboxyl N found		
	mg.	<i>c.mm.</i> 0.07138 N HCl (N/14)	<i>c.mm.</i> 0.07138 N HCl	<i>c.mm.</i> 0.07138 N HCl	mg.	per cent of substance	per cent of theory
25 cc. flask	0.4642 alanine	190.5	46.7	143.8	0.0719	15.49	98.5
		190.5	44.7	145.8	729	15.70	99.9
		190.5	45.7	144.8	724	15.60	99.2
		190.5	43.2	147.3	737	15.88	101.0
		174.6	31.6	143.0	715	15.40	98.0
		174.6	30.6	144.0	720	15.51	98.7
Average.....							99.2
Mean deviation from average.....							$\pm 0.6$
10 cc. flask	0.4618 alanine	181.3	38.6	142.7	0.0714	15.46	98.4
		181.3	35.2	146.1	732	15.85	100.8
		181.3	36.8	144.5	723	15.66	99.6
		181.3	38.2	143.1	716	15.50	98.6
		181.3	37.8	143.5	718	15.55	98.9
		177.5	33.9	143.6	718	15.55	98.9
Average.....							99.2
Mean deviation from average.....							$\pm 0.7$

#### SUMMARY

A titration method is described for determining free amino acids by titration of the  $\text{CO}_2$  evolved from their carboxyl groups during reaction with ninhydrin. The only special apparatus required consists of two 25 cc. Erlenmeyer flasks connected by a U-tube. The reaction occurs in one flask; the  $\text{CO}_2$  then distils *in vacuo* during 2 to 3 minutes into standard barium hydroxide in the other flask, where the excess hydroxide is titrated. The same apparatus serves for macro- and microanalyses.

For amounts of carboxyl carbon above 0.4 mg. the mean variability of results is of the order of  $\pm 0.3$  per cent of the amounts measured. Microanalyses with samples down to 0.04 mg. of carboxyl carbon can be done with a mean error under 1 per cent.

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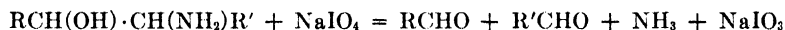
## THE DETERMINATION OF HYDROXYLYSINE IN PROTEINS\*

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In the present procedure hydroxylysine is precipitated from protein hydrolysates with the other diamino acids by means of phosphotungstic acid. In the mixture of the diamino acids hydroxylysine is determined by means of the ammonia liberated by the reaction



The peculiar ability of periodic acid to split an aliphatic chain between two adjacent carbons bearing groups with active hydrogens was applied by Malaprade (2) to the  $\cdot\text{CH(OH)·CH(OH)·}$  group in polyalcohols. Nicolet and Shinn (8) were the first to apply it to hydroxyamino acids. They showed that serine and threonine were split with formation of formaldehyde and acetaldehyde respectively, and, with regard to ammonia, stated, "The progress of  $\text{HIO}_4$  consumption with time is entirely consistent with the assumption that the other direct products from serine are (as would be expected) ammonia and glyoxylic acid." The present authors (21) applied the reaction to hydroxylysine, and found that if the periodate acted in alkaline solution the hydroxylysine was split with formation of 1 mole of formaldehyde and 1 mole of ammonia, and the reduction of 1 mole of  $\text{IO}_4^-$  to  $\text{IO}_3^-$ .

\* Measurement of the ammonia produced by alkaline periodate as a means of determining hydroxylysine and other hydroxyamino acids with the  $\cdot\text{CH(OH)·CH(NH}_2\text{)·}$  group has been mentioned in a preliminary note (21). The present paper contains details necessary for precision, and for the application of the method to hydroxylysine determination in protein hydrolysates.

In preliminary experiments, when we treated hydroxylysine and serine with free periodic acid and titrated the unused excess of periodic acid in acid solution with thiosulfate, as was done by Malaprade (2), we were unable either to demonstrate a quantitative reaction with hydroxylysine or to confirm the rapid course noted by Nicolet and Shinn (8) with serine. When, however, the excess periodate was titrated with arsenite by the method of Müller *et al.* (5, 6) and of Fleury and Lange (1),<sup>1</sup> which includes preliminary alkalization with excess  $\text{NaHCO}_3$ , we obtained immediate reduction of 1 mole of  $\text{IO}_4^-$  to  $\text{IO}_3^-$  by either amino acid.

It developed that the reaction of the hydroxyamino acids was not completed during the treatment with the acid solution of periodic acid (*e.g.*, see lowest curve of reaction with hydroxylysine, Fig. 2; serine yields practically identical curves), but was instantly completed at the moment when the bicarbonate was added to prepare the solution for the arsenite titration. This observation led us to study the conditions of alkalinity which would most sharply differentiate the reactivity of hydroxylysine from that of the non-hydroxyamino acids, and to the adoption of conditions for quantitative ammonia production by the reaction. It was found that although the slightly alkaline reaction of  $\text{NaHCO}_3$  sufficed for a quantitative reaction of periodate with the  $\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot$  group, the presence of free  $\text{NaOH}$  was necessary in order to prevent low results for ammonia.

It was found furthermore that if periodate reacts in a solution containing only hydroxyamino acids, the yields of ammonia are only 80 to 90 per cent of the theoretical, presumably because part of the ammonia condenses with some of the aldehyde that is formed from the rest of the hydroxyamino acid. If a sufficient excess of non-hydroxyamino acid is present, however, a quantitative yield is obtained from the hydroxy acids, presumably because the amino groups of the non-hydroxyamino acids condense with the nascent aldehyde before it can combine with the ammonia. Consequently, in analyses of hydroxylysine, an adequate concentration of glycine is added in order to make the ammonia yield quantitative.

<sup>1</sup> We thank Dr. Nicolet for his courtesy in informing us, prior to detailed publication, concerning his use of this titration in the experiments of the preliminary note of Nicolet and Shinn (8).

Since hydroxylysine is decomposed by alkaline periodate with formation of 1 molecule each of formaldehyde and ammonia, and the reduction of 1 molecule of periodate to iodate, three ways are possible for quantitative determination of this amino acid; *viz.*, measurement of the reduced periodate, of the generated formaldehyde, or of the ammonia.

Titration of the amount of periodate reduced proved to be a convenient method for analysis of preparations of isolated hydroxylysine salts for their analytical purity.<sup>2</sup> Such titration, however, is not sufficiently specific to measure hydroxylysine in mixtures of the diamino acids, for cystine, as shown by Nicolet and Shinn (8), and histidine, as found by ourselves, also reduce periodate by reactions other than that formulated above. They do not yield ammonia.

Precipitation of the formaldehyde as the dimedon compound by Vorländer's method (23), used in determining the structure of hydroxylysine (21), can be applied to a few mg. of hydroxylysine: it is necessary merely to acidify the periodate reaction mixture with acetic acid to pH 5 or 6, add an excess of dimedon, let stand overnight for precipitation, and weigh the washed precipitate after drying it in a desiccator. It may be the most convenient method of analysis when only occasional determinations are to be done.

For series of analyses, however, determination of the ammonia has proved most convenient, and has been used for the protein analyses reported in this paper.

Separation of the diamino acids from the monoamino fraction was found to be a necessary preliminary to determination of hydroxylysine by the yield either of formaldehyde or of ammonia. In the monoamino fraction serine has been shown by Nicolet and Shinn (8, 9) to yield formaldehyde, and the authors have found that ammonia is produced quantitatively by all the  $\beta$ -hydroxy-amino acids, serine, threonine, and  $\beta$ -hydroxyglutamic acid (21). Hence it is necessary to separate these amino acids from hydroxylysine before the latter is determined.

The present hydroxylysine determination is based on the as-

<sup>2</sup> For a microanalysis 4 or 5 mg. of hydroxylysine monochloride, or twice as much picrate, are dissolved in 3 cc. of water, and treated with 2 cc. of 0.02 M  $\text{HIO}_4$ , of which the excess is then titrated with arsenite as described for "Alkaline solutions" (see p. 694).

sumption that this amino acid is the only one in the phosphotungstate precipitate which yields ammonia by reaction with periodate. This assumption has been verified for the known diamino acids, arginine, histidine, lysine, and cystine. The possibility remains that some other hydroxy derivative of the diamino group may exist unidentified in proteins, as did hydroxylysine until recently (20), and may be precipitated and determined with the hydroxylysine. The possibility appears small, and we report the method and the results as applying to hydroxylysine, with the reservation that the figures may include some other, as yet unknown, basic hydroxy-amino acid with similar properties.

Demonstration in the preliminary note by the authors and Hastings and Klemperer (21) that alkaline periodate evolves ammonia quantitatively from the hydroxyamino acids as a class (except hydroxyproline), and not from other types of amino acids yielded by protein hydrolysis, indicated the possibility of determining the total amounts of hydroxyamino acids in protein hydrolysates by the ammonia procedure. It has since been employed for this purpose by Martin and Synge (3), by Nicolet and Shinn (10, 11, 13), and by Nicolet and Saidel (7). The details described below, which we have found essential for precision in applying the ammonia procedure to hydroxylysine, serve also for the other hydroxyamino acids with the  $\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot$  group.

#### DETERMINATION OF HYDROXYLYSINE IN PROTEINS

##### *Apparatus*

The apparatus used in the analysis of proteins by the nitrogen distribution method (16, 17), for the hydrolysis of small portions of protein, distillation of HCl and ammonia *in vacuo*, and precipitation and washing of the phosphotungstates of the diamino acids.

The apparatus for determination of ammonia by aeration, described by Van Slyke and Cullen ((19), and (12) p. 548).

A gas flowmeter or gas meter to measure the air current used for transfer of ammonia by aeration.

##### *Reagents*

6 *N* HCl, made by diluting 1 volume of concentrated HCl with 1 volume of water.

*Calcium hydroxide*, finely divided light powder.

*Phosphotungstic acid*, purified by Winterstein's method (24) applied as follows: The acid is dissolved in an equal weight of water and shaken in a separatory funnel with enough ether to cause formation of three layers, the lowest being ether containing the desired fraction of the acid, the middle being aqueous, and the top the excess ether. The lowest layer is washed three times, each time with an equal volume of water. The heavy ether solution is then transferred to a porcelain dish and dried on a steam bath. The product is not hygroscopic and forms a clear solution. The recovery was 70 per cent when Merck's Reagent grade was used.

*Sulfuric acid*, approximately 0.1 N.

*Potassium carbonate*, saturated solution.

*Sodium hydroxide*, approximately 2 N, 5 N, and 10 N.

*Glycine solution*, 5 gm. per 100 cc.

*Caprylic alcohol*, redistilled, middle portion.

*Periodic acid*, 0.2 M. The theoretical amount of crystalline  $\text{HIO}_4 \cdot 2\text{H}_2\text{O}^3$  is 45.59 gm. per liter, but the hygroscopic character of the crystals prevents accurate weighing. 46 gm. are weighed out roughly and diluted to 1 liter with water. A slight precipitate may settle on standing. It is best to let the solution stand overnight or longer, and filter before using. It is standardized against 0.1 N arsenite solution, made according to Treadwell and Hall (15).

*Bromine solution*. 60 gm. of KBr are dissolved in 100 cc. of water, and 2.5 cc. of bromine are dissolved in the KBr solution (22). The solution is kept in a glass-stoppered bottle which is opened as little as possible. The bromine content is checked at least once a month by titration with thiosulfate, as it gradually becomes weaker from volatilization. If loss exceeds 20 per cent, the solution is discarded or reinforced with added bromine.

#### PROCEDURE

##### *Hydrolysis of Protein and Preparation of Solution of the Diamino Acids*

The analysis can be carried through with as little as 0.5 gm. of protein, but in the analyses reported in this paper 3 gm. portions

<sup>3</sup> Crystalline periodic acid can be obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio (14).



were hydrolyzed, giving sufficient material for repetition of duplicates and for testing the diamino acid solution for the presence of ammonia.

The protein is hydrolyzed by boiling under a reflux for 24 hours with about 30 times its weight of 6 N HCl. The free HCl is driven off *in vacuo*, the ammonia removed by distillation with calcium hydroxide *in vacuo*, as described by Van Slyke (16, 17), and the melanin adsorbed and removed with the calcium hydroxide (16, 17).

In the ammonia-free residue the diamino acids are precipitated with phosphotungstic acid, as described in the above publications (16, 17), except that the precipitation is done in larger volume and with a lesser concentration of HCl present. It has been found, in work which will be published shortly, that if the concentration of HCl is reduced from 1 N, originally used (16, 17), to 0.25 N, the solubility of the diamino acid phosphotungstates is markedly diminished, so that their precipitation can be carried out in larger volume, with less danger of occlusion of significant amounts of the monoamino fraction. Accordingly, when 3 gm. of protein are hydrolyzed, the precipitation is done with 25 gm. of phosphotungstic acid in a volume of 300 cc. (at 23–25°) and in the presence of 0.25 N HCl. The precipitate is filtered at the end of 48 hours, and washed with suction (17) five times with 8 cc. portions of a solution which contains 50 gm. of phosphotungstic acid dissolved in 1 liter of 0.25 N HCl.

In order to increase the assurance that the diamino acids are freed from the monoamino fraction, the phosphotungstates are recrystallized as follows: The washed precipitate is rinsed with water back into the precipitation flask, and is redissolved by addition of 2 N sodium hydroxide until the solution is neutral to alizarin red. The solution is then made up to 300 cc. volume again, 6 cc. of concentrated HCl are added to make the normality approximately 0.25, the solution is heated, and 15 gm. of phosphotungstic acid are added. At the end of 48 hours the precipitate is again filtered and washed (17).

The precipitate of diamino acids thus obtained is rinsed into a 30 cc. volumetric flask, a drop of alizarin red is added, and 5 N sodium hydroxide, with stirring, until the precipitate dissolves and the solution is neutral to the indicator. The volume is then made up to 30 cc.

*Treatment of Diamino Acid Solution with Periodate and Aeration of Ammonia Formed from Hydroxylysine*

The analysis is carried out on duplicate 5 cc. portions of the diamino acid solution.

The Van Slyke-Cullen aeration apparatus is used for both the generation of the ammonia and its transfer to acid by aeration, two pairs of tubes being required when the analysis is run in duplicate.

Into each of the two receiving tubes are measured accurately 12 cc. of the approximately 0.1 N sulfuric acid. No caprylic alcohol is added to these tubes. It is preferable not to have it present when the ammonia is determined gasometrically by the hypobromite reaction.

Into each of the two generating tubes for the periodate reaction are measured the following, *in the order given*:

5 cc. of the diamino acid solution. (It is desirable that the sample contain an amount of hydroxyamino acid to yield from 0.1 to 2 mg. of ammonia nitrogen.)

1 cc. of the 5 per cent glycine solution.

1 drop of caprylic alcohol.

1 cc. of 2 N NaOH.

2 cc. of 0.2 M periodic acid.

10 cc. of the saturated  $K_2CO_3$  solution.

Ammonia is formed as soon as the periodic acid is added. To prevent any loss of the ammonia the carbonate is added to each tube at once after the periodic acid, and the tube is then quickly closed with the stopper bearing the inlet and outlet tubes for the air current. As soon as all the generating tubes have been filled, they are connected in series and the air current is started through them. It is run slowly at first, about 1 bubble per second, for 2 minutes; then it is gradually accelerated to reach 4 liters per minute at the end of 5 minutes. The current is drawn through at this rate for 25 minutes, so that 100 liters are passed. Several sets of tubes can be thus aerated in series together.

*Manometric Determination of Aerated Ammonia*

Of the 12 cc. of solution in the receiving tube 10 cc. are transferred with a rubber-tipped pipette to the chamber of the Van Slyke-

Neill manometric apparatus. To remove the air from the solution the mercury is lowered to the bottom of the chamber, the latter is shaken for 2 minutes, and the extracted air is ejected. This process is once repeated to remove the last traces of air. Then 1.25 cc. of the 10 N NaOH solution and 0.75 cc. of the bromine solution are mixed in the cup of the chamber and 1.5 cc. of the mixture are drawn down into the chamber (22).

The mercury in the chamber is lowered to the 50 cc. mark, and the chamber is shaken 3 minutes to extract the nitrogen formed by action of the hypobromite on the ammonia. The gas volume is brought to either 2 or 0.5 cc., according to the amount present, and  $p_1$  is read on the manometer. The gas is then ejected, the water meniscus returned to the 0.5 or 2 cc. mark, and  $p_0$  is read on the manometer.

*Blank Analysis to Determine the c Correction*—An analysis, including the aeration, is carried through in the same way outlined above, except that the 5 cc. of unknown amino acid solution are replaced by 5 cc. of ammonia-free water. The  $p_1 - p_0$  value obtained is the  $c$  correction for the reagents.

### *Calculation of Gasometric Analyses*

To calculate the amount of ammonia nitrogen yielded by the sample of amino acids the formula is

$$\text{Mg. ammonia N} = 1.2 (p_1 - p_0 - c) \times F_1 \times F_2$$

The factor  $F_2$  is used only when the ammonia nitrogen is less than 0.5 mg.

When the hydroxy acid determined is hydroxylysine, the ammonia nitrogen obtained is multiplied by 2 to give total hydroxylysine nitrogen.

The factor 1.2 corrects for the fact that of the 12 cc. of 0.1 N acid in the receiving tube only 10 cc. were taken for the manometric determination.  $c$  is the  $p_1 - p_0$  value obtained in the blank analysis.

$F_1$  is the factor for calculating ammonia nitrogen from the pressure of the  $N_2$  yielded by the hypobromite reaction given in Table I of Van Slyke's paper (18) and by Peters and Van Slyke ((12) p. 358). The factors given in these references are for pressure values meas-

ured with the gas at 2 cc. volume. If the measurement is made with the gas at 0.5 cc. volume, the 2 cc. factors are divided by 4.

The factor,  $F_2$ , corrects for the fact that when the amount of ammonia reacting with hypobromite is below 0.5 mg. the yield of  $N_2$  per mg. of ammonia, as shown by Table I, is lower than indicated by  $F_1$ . The values of  $F_2$  are given by Fig. 1.

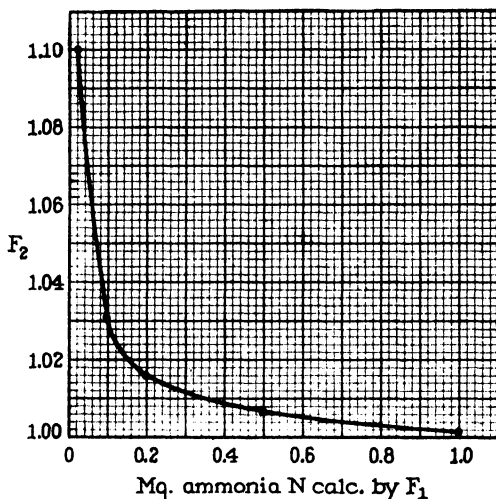


FIG. 1. Values of calculation factor  $F_2$  for varying amounts of ammonia determined by hypobromite.

#### *Alternative Measurement of Aerated Ammonia by Titration*

If less than 1.5 mg. of ammonia nitrogen is determined, absorption and titration in boric acid solution, introduced by Meeker and Wagner (4), can be applied. It is convenient, because it obviates the use of standard alkali and affords a direct titration instead of one by difference. If more than 1.5 mg. of ammonia nitrogen is present, however, some is likely to escape absorption by the boric acid.

For the boric acid procedure, the receiving tube is charged with 15 cc. of 2 per cent boric acid and 1 drop of 0.1 per cent brom-cresol green solution. After aeration the solution is titrated with  $N/70$  sulfuric or hydrochloric acid from a 10 cc. burette. The end-point is the color of a control tube with 15 cc. of 2 per cent boric acid

plus an amount of water equal in volume to the N/70 acid added in the titrated tube.

If more than 1.5 mg. of ammonia is present, the original Van Slyke-Cullen procedure is followed. The receiver tube is charged with 15 or 25 cc. of N/70 acid, and the excess acid is titrated back with standard NaOH, with alizarin red as indicator.

Control analyses for ammonia present in the reagents are carried out, as for the gasometric determination, and the correction, *c*, is expressed in cc. of N/70 acid.

The calculation is

$$\text{Mg. ammonia N} = 0.2 (\text{cc. N/70 acid} - c)$$

In so far as the titration and manometric measurements of the aerated ammonia compare, the authors somewhat prefer the manometric. The manometric method is immune to error from the carrying over of traces of alkaline spray from a generator tube to a receiving tube, and it permits more precise measurement of quantities in the lower ranges.

*Error Due to Solubility of Hydroxylysine Phosphotungstate*—In mixtures of the diamino acids precipitated by phosphotungstic acid the diamino acids are precipitated in part at least as mixed salts (data to be presented later), and not as individual substances each with its own independent solubility. The effect on a given diamino acid present in small amount, or forming a more soluble phosphotungstate than the average of the group, is to diminish the loss of such diamino acid below the loss that would be calculated from the solubility of its isolated phosphotungstate. Thus histidine or hydroxylysine may be present in such small amount that according to the solubilities of their isolated phosphotungstates neither of them should be precipitated at all; yet precipitation is fairly complete if plenty of lysine or arginine is present. The scantily present histidine or hydroxylysine is carried down with the precipitate of the other basic amino acids.

As shown in the experimental part, in a mixture of lysine and hydroxylysine precipitated by phosphotungstic acid the amount of hydroxylysine in a liter of the filtrate varies inversely as the ratio of lysine to hydroxylysine in the precipitate. Hydroxylysine phosphotungstate by itself dissolves at 20° to the extent of about 18 mg. of nitrogen per liter of phosphotungstic acid solution.

Mixed with 10 times the amount of lysine, the hydroxylysine is carried down so completely that only 3 mg. of its nitrogen remain in solution per liter. The 600 cc. of phosphotungstic acid solution used in the precipitation and recrystallization of the diamino acids from 3 gm. of protein in the present hydroxylysine determination would suffice at 20° to dissolve 11 mg. of hydroxylysine nitrogen if this amino acid were alone in the precipitate. Yet Table IX shows that when 2.5 mg. of hydroxylysine nitrogen were added to hemoglobin, a protein which contains none, 2.2 mg. were recovered from the recrystallized phosphotungstates, only 0.3 mg., equivalent to 0.06 per cent of the protein nitrogen, being lost in the mother liquors. When the same amount of hydroxylysine was added to gelatin (Table IX), the loss was 0.6 mg., equivalent to 0.12 per cent of the protein nitrogen. In gelatin hydroxylysine is present to the extent of about 1 per cent of the protein nitrogen, forms a larger part of the hexone base precipitate than in the hemoglobin, and correspondingly there was a greater absolute loss of hydroxylysine in the mother liquors.

The conclusion is that one cannot apply a simple solubility correction to hydroxylysine phosphotungstate, but that if the diamino acids in a protein are present in sufficient amounts to form an abundant precipitate (*e.g.*, if their nitrogen exceeds 10 per cent of the protein nitrogen), one may assume that most of the hydroxylysine will be carried down with the precipitate of the other diamino acids, even if the hydroxylysine itself contains less than 1 per cent of the protein nitrogen.

If a protein is encountered with less than 10 per cent of its nitrogen in diamino acids, it will be desirable, if hydroxylysine is determined by the present method, to add enough arginine or lysine (tested for freedom from hydroxyamino acid by the periodate reaction) to bring the nitrogen precipitable by phosphotungstic acid up to 15 or 20 per cent of the total protein nitrogen, in order to provide a precipitate that will carry down with it the hydroxylysine.

*Correction for Preformed Ammonia in Amino Acid Solutions Analyzed*

In the procedure used in this paper for preparation of the diamino acid solution ammonia is removed from the hydrolysate

by distillation *in vacuo* before the phosphotungstates of the diamino acids are precipitated. However, if the removal should not be complete, the ammonia left in the hydrolysate would be more or less completely precipitated with the phosphotungstates of the diamino acids, and would be measured as hydroxylysine by the above method. Also, after the solution of the diamino acids has been prepared, if it is permitted to stand about for 1 or more days in a warm room before it is analyzed, there is a possibility of ammonia formation from decomposition of some of the material.

If there is any possibility of the presence of ammonia in the diamino acid solution, a portion of 5 cc. is analyzed for free ammonia by mixing 5 cc. in a generating tube of the Van Slyke-Cullen apparatus with 5 cc. of saturated potassium carbonate solution and a drop of caprylic alcohol, and aerating and determining the ammonia, by either gasometric measurement or titration. The blank in this case is run on 5 cc. of the carbonate solution plus 5 cc. of ammonia-free water. Any preformed ammonia found in the amino acid solution is subtracted from the hydroxylysine value.

We have performed this control with the diamino acid solution of all the proteins reported in this paper, but have not in any case found significant amounts of preformed ammonia.

*It is not permissible to remove preformed ammonia by a preliminary aeration with half saturated  $K_2CO_3$  solution before the periodic acid is added.* In the case of serine such preliminary treatment was found to alter the hydroxyamino acid in such a manner that part of it became non-reactive with periodate. Thus, after 22 minutes preliminary aeration, which did not evolve any ammonia from the serine, addition of periodate gave only 92 per cent of the theoretical ammonia; and when the preliminary aeration was for 50 minutes, treatment with periodate yielded only 86 per cent. We have not duplicated these experiments with hydroxylysine, but they suffice to indicate the need of caution in treating the  $\cdot CH(OH) \cdot CH(NH_2) \cdot$  group with strong alkali. Preformed ammonia may either be removed by vacuum distillation from mildly alkaline solution at low temperature, as described for the determination of hydroxylysine, or it may be determined in a control analysis without periodate, as described above, and subtracted from the total ammonia found by aeration with periodate.

*Remarks Concerning Conditions for Periodate-Ammonia Reaction*

While a definitely alkaline reaction is necessary for the reaction, addition of too much NaOH would retard it. If 3 cc., instead of 1 cc., of the 2 N NaOH are added, the yield of ammonia is reduced below the theoretical (Table IV).

It is desirable not to prolong the aeration greatly beyond the prescribed 25 or 30 minutes. In the mixture of reaction products, glycine, and periodate, a very slow secondary evolution of ammonia from the glycine occurs. In experiments with serine it amounted only to 0.2 per cent of the serine nitrogen when the aeration was continued for a second half hour, but this slow reaction continued, and if the reagents were left together for hours before the aeration was finished the error would become appreciable.

In the ammonia aeration method as originally described by Van Slyke and Cullen (19), it was permissible to add the  $K_2CO_3$  either in solid form or in saturated solution; the essential was to get the aerated solution at least half saturated with  $K_2CO_3$ . In the present periodate-ammonia method *it is not permissible to use the solid  $K_2CO_3$*  for the reason that it heats the solution up to about  $70^\circ$  and causes an appreciable evolution of ammonia from the glycine.

## EXPERIMENTAL

*Effects of Acidity and Alkalinity on Reduction of Periodate by Hydroxylysine*

In order to ascertain the optimal conditions for reaction of periodate with the  $\cdot CH(OH) \cdot CH(NH_2) \cdot$  group, the time curves of the reaction were plotted by following titrimetrically the reduction of the periodate. Control experiments were performed in the same way with alanine and serine.

The reacting solutions contained hydroxylysine monochloride in 0.005 M concentration and  $IO_4^-$  in initial concentration of 0.015 to 0.016 M. To some of the hydroxylysine solutions only  $HIO_4$  was added, so that the acidity was that of the 0.015 M periodic acid, somewhat reduced by the buffer effect of, and reaction with, the amino acid. In other solutions  $H_2SO_4$  or NaOH was added, as indicated in Fig. 2.



At intervals after the solutions were prepared, periodic acid being the last constituent added, samples were drawn and titrated for unreduced periodate. For the alkaline solutions the arsenite titration was used, and for acid solutions the thiosulfate titration. The results are shown in Fig. 2.

The technique used in the titrations was the following:

*Alkaline Solutions*—The titrations were performed with 0.02 N arsenite solution, which titrates an equal volume of 0.01 M  $\text{NaIO}_4$ . The 0.02 N arsenite was made fresh on the day used by mixing 1 volume of 0.1 N stand-

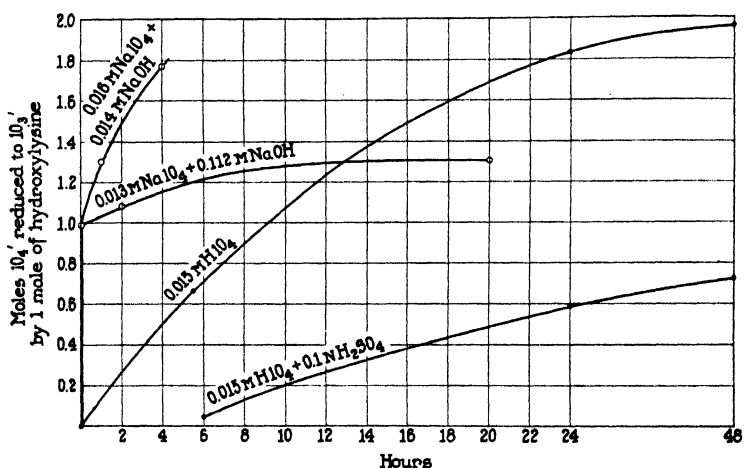


FIG. 2. Reduction of periodate by hydroxylysine in solutions of different alkali and acid content.

and arsenite solution with 1.25 volumes of 1 M  $\text{NaHCO}_3$  solution and diluting to 5 volumes with water.

For the titration a 5 cc. portion of the amino acid-periodate reaction mixture was withdrawn and treated with 5 cc. of 1 M  $\text{NaHCO}_3$  (unless the mixture had already been made up with  $\text{NaHCO}_3$ ) and 1 cc. of 20 per cent KI solution. When the bicarbonate is added, 1 mole of periodate per mole of hydroxyamino acid is instantly reduced to iodate, and then secondary reactions slowly continue reducing the periodate, at the rate of about 0.01 mole per minute per mole of hydroxy acid (see top curve of Fig. 2). To avoid significant plus error from the secondary reactions, it was necessary to start the titration with arsenite at once and to finish it without delay. An alternative procedure was to add excess arsenite before the KI, and titrate back with iodine solution, but this indirect titration was unnecessary if the arsenite titration was carried through quickly.

*Acid Solutions*—5 cc. of the amino acid-periodate mixture were mixed with 5 cc. of 1 N hydrochloric acid, 1 cc. of 20 per cent KI solution, and 10 cc. of water. The titration was performed with 0.1 N thiosulfate, of which 8 equivalents titrate 1 mole of periodate.

Hydroxylysine in the two alkaline solutions (Fig. 2) consumed 1 atom of periodate oxygen instantly. Reaction with a 2nd molecule of periodate occurred at a rate inversely proportional to the alkalinity. In acid solution the reaction was immensely retarded, and there was no sharp break between the velocities with which the 1st and the 2nd periodate oxygen atoms were consumed.

The ammonia production has been found in other experiments to occur quickly, with the utilization of the 1st oxygen atom. It appears that in alkaline solution the typical reaction, by which the  $\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}(\text{OH})\cdot$  group is split, occurs instantly, and that the products consume a 2nd atom of oxygen at a rate which is much slower, and decreases with increasing alkalinity.

Control experiments with serine gave curves identical with those of hydroxylysine. Alanine did not react measurably in 6 hours, in either acid or alkaline solution.

#### *Recovery of Small Amounts of Ammonia by Manometric Hypobromite Method*

Standard solutions of ammonium sulfate were prepared in 0.1 N sulfuric acid of such strength that 10 cc. contained respectively 1.0, 0.5, 0.2, and 0.1 mg. of ammonia nitrogen. The ammonia contents were determined by pipetting 10 cc. portions directly into the chamber of the manometric apparatus of Van Slyke and Neill, and analyzing with hypobromite as described above for hydroxylysine determinations. The results calculated with the nitrogen factors of Van Slyke (18, 12) are shown in Table I. The results show that as the amount of nitrogen determined sinks below 1 mg. the factor must be increased to give 100 per cent values. From these data the  $F_2$  factors of Fig. 1 are plotted.

#### *Destruction of Ammonia by Free Periodic Acid*

A solution of ammonium sulfate was prepared such that 5 cc. contained 0.986 mg. of ammonia nitrogen. 5 cc. portions of this solution were treated as prescribed for the diamino acid solution in the hydroxylysine determination, except that the amount of periodic

acid was varied, and no NaOH or glycine was added. The results (Table II) show incomplete recoveries of the ammonia when periodic acid was added. The amounts of  $\text{HIO}_4$  added were below and above the 0.4 mM used in the hydroxylysine analyses. It appears that when ammonia and periodic acid are mixed in acid

TABLE I  
*Recovery of Ammonia from Ammonium Sulfate by Gasometric Hypobromite Method*

Amount of nitrogen in sample analyzed	Recovery, average of 5 analyses*	Maximum deviation from average	Mean deviation from average
mg.	per cent	per cent	per cent
1.0	99.8	$\pm 0.1$	$\pm 0.1$
0.5	99.3	$\pm 0.6$	$\pm 0.3$
0.2	98.4	$\pm 0.4$	$\pm 0.2$
0.1	96.9	$\pm 0.5$	$\pm 0.4$
0.02	90.9	$\pm 4.7$	$\pm 1.9$

\* Calculated by  $F_1$ , without use of  $F_2$ .

TABLE II  
*Recovery of Ammonia from Ammonium Sulfate by Aeration with Half Saturated Potassium Carbonate with and without Previous Addition of Free Periodic Acid*

Periodic acid added	Ammonia nitrogen recovered
mM	per cent
0	100.3
0.18	98.4
	98.7
0.54	93.9
	93.5

solution measurable destruction of the ammonia occurs, presumably in the interval before the potassium carbonate is added.

#### *Non-Destruction of Ammonia in Alkaline Periodate*

The above experiment was repeated, but when periodic acid was added, it was preceded by sodium hydroxide, so that the periodate touched the ammonia only in alkaline solution. The

results in Table III indicate that when the periodic acid was added in the presence of excess NaOH there was a quantitative recovery of the ammonia by subsequent aeration.

TABLE III  
*Recovery of Ammonia from Ammonium Sulfate. Effect of NaOH in Preventing Destruction of Ammonia by Periodic Acid*

Sodium hydroxide added	Periodic acid added	Ammonia nitrogen recovered
<i>mM</i>	<i>mM</i>	<i>percent</i>
0	0	100.1
0.6	0.18	98.7 99.5
2.0	0.54	100.6 99.7

TABLE IV  
*Effect of Sodium Hydroxide on Yield of Ammonia from Reaction of Serine and Periodate*

2 N NaOH	Percentage of theoretical $\text{NH}_3$ yield	
	By titration	Manometric
<i>cc.</i>		
3.0	88.5 88.2	90.1
2.0	100.1 100.8	100.6
1.0	99.6 99.3	99.4
0.5	94.2	94.7
0	93.6 93.6	93.8

*Effect of Varying Amounts of NaOH on Yield of Ammonia from the Reaction of Serine with Periodate*—A solution of serine was used of which 5 cc. contained 26.50 mg. of the amino acid. Analyses

of 5 cc. aliquots were carried out as described for the diamino acid solution, with the following exceptions: (a) The amount of 2 N NaOH was varied; (b) the aeration was into 15 cc. of 0.02 N sulfuric acid. After the aeration 5 cc. of the solution in the receiver were used for manometric determination of the ammonia, and in a second 5 cc. portion the excess acid was titrated back with 0.02 N NaOH. The results are given in Table IV. They show that quantitative yields of ammonia were obtained when the amount of 2 N NaOH added was 1 or 2 cc., but that use of either more or less depressed the yield.

TABLE V  
*Accuracy of Determination of Serine by Estimation of Ammonia  
Liberated by Reaction with Alkaline Periodate*

Amount of nitrogen in sample analyzed	Recovery, average of 6 analyses	Maximum deviation from average	Mean deviation from average
<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.0	100.3	$\pm 0.8$	$\pm 0.4$
0.5	99.8	$\pm 0.6$	$\pm 0.3$
0.2	99.5	$\pm 2.8$	$\pm 1.8$
0.1	100.0	$\pm 2.4$	$\pm 1.4$

*Estimation of Varying Quantities of Serine by Technique Used for  
Hydroxylysine*

In order to test the accuracy of the technique with varying amounts of material, without sacrificing the expensively prepared hydroxylysine, series of analyses were run on standard solutions of serine. This substitution appeared justified because experiments with serine, like those with hydroxylysine charted in Fig. 2, showed reaction curves with periodate which were identical with those of hydroxylysine.

Standard solutions of serine were prepared such that 5 cc. contained 1.0, 0.5, 0.2, and 0.1 mg. of serine nitrogen. 5 cc. portions of these solutions were treated by the technique described for analysis of the diamino acid solutions for hydroxylysine, and the hydroxyamino nitrogen values were calculated by the formula for the manometric analysis, with the use of  $F_2$  from Fig. 1 in estimating the nitrogen values of 0.5 mg. and less. The results are shown in Table V.

*Ammonia Liberated by Alkaline Periodate from Different Types of Amino Acids<sup>4</sup>*

By the technique described for analyzing the diamino acid solution, the following non-hydroxyamino acids were found to yield no ammonia: arginine, aspartic acid, cystine, cysteine, djenkolic acid, glutamic acid, glycine, histidine, homocystine, leucine, lysine, methionine, phenylalanine, proline, tryptophane, tyrosine, valine. *No amino acid without the  $\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot$  group evolved under the conditions of the analysis enough ammonia to amount to 0.5 per cent of its total nitrogen.*

TABLE VI  
*Ammonia Liberated by Alkaline Periodate from Hydroxyamino Acids*

Substance	Nitrogen in sample analyzed	Ammonia liberated
	mg.	per cent of theoretical
$\beta$ -Hydroxyaspartic acid	2.887	98.3
$\beta$ -Hydroxyglutamic " hydrochloride (synthetic)	0.931	99.3
Hydroxylysine monohydrochloride	0.463	99.4
Hydroxyproline	1.680	0
Phenylserine	1.039	100.3
Serine	See Table V	
Threonine	0.527	100.6

On the other hand, all the amino acids which did contain this group evolved quantitatively as ammonia the nitrogen from the  $\text{NH}_2$  (Table VI). Hydroxyproline alone among the hydroxyamino acids in Table VI evolved no ammonia, a behavior in accordance with the accepted assumption that the hydroxyl is not in the  $\beta$  position in this amino acid. It follows from these results that before hydroxylysine can be determined in a protein hydrolysate by the periodate-ammonia method, the hydroxylysine must be separated from the threonine, serine, and  $\beta$ -hydroxyglutamic acid; a separation accomplished by the phosphotungstic acid precipitation.

<sup>4</sup> For the methionine, cysteine, homocystine, and djenkolic acid we thank Dr. Vincent du Vigneaud, and for the synthetic hydroxyglutamic acid, Dr. H. D. Dakin.

*Non-Effect of Phosphotungstic Acid on Liberation of Ammonia by Periodate*—A serine standard was prepared in a 20 per cent phosphotungstic acid solution which had been neutralized with NaOH in the same manner as in the preparation of the base solutions for analysis of protein hydrolysates for hydroxylysine described above. Each 5 cc. sample contained 0.45 mg. of serine nitrogen, 1 gm. of phosphotungstic acid, and sufficient alkali to neutralize the solution. The yields of ammonia from the serine were theoretical.

*Ammonia Liberated by Alkaline Periodate from Ethanolamine, Glucosamine, and Urea*

Ethanolamine and glucosamine have the  $\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot$  group which in the hydroxyamino acids causes quantitative libera-

TABLE VII  
*Ammonia Liberated by Alkaline Periodate from Glucosamine, Ethanolamine, and Urea*

Substance	Nitrogen in sample analyzed	Ammonia liberated
	mg.	per cent of total N
$\alpha$ -Glucosamine hydrochloride .....	0.705	32.2
Ethanolamine " .....	1.606	85.0
Urea .....	7.00	0

tion of the nitrogen as ammonia. Urea does not have this group, but yields ammonia so readily under various conditions that it was desirable to test its behavior with periodate. The results obtained with these three substances are shown in Table VII. The reactions with ethanolamine and glucosamine were unexpectedly incomplete, but have been repeated, and the substances have been reanalyzed and found analytically pure. We have no explanation for the incompleteness of the liberation of the ammonia by these two substances.

*Solubility of Hydroxylysine Phosphotungstate Precipitated together with Varying Proportions of Lysine Phosphotungstate*

Portions of 400 mg. of mixed picrates of lysine and hydroxylysine in varying proportions were dissolved in 220 cc. of 0.25 N hydro-

chloric acid, 13 gm. of purified phosphotungstic were added, the solution was heated to 100°, and then was left 48 hours at 20° for the phosphotungstates to crystallize. The solution was then filtered with suction, without washing. Of the filtrate 200 cc. were freed of phosphotungstic acid by extraction with 1:1 amyl alcohol-ether mixture (17), the aqueous phase was concentrated to dryness to remove HCl, and was brought to 11 cc. volume. Portions of 5 cc. were used for determination of hydroxylysine nitrogen by the periodate-ammonia method described in this paper.

Blank analyses were done in which the same procedure was carried out with all the reagents except the diamino acids. The amount of ammonia nitrogen yielded by the blanks was 0.043 mg.,

TABLE VIII

*Concentrations of Hydroxylysine Nitrogen in Filtrates from Mixed Phosphotungstates of Lysine and Hydroxylysine*

Temperature, 20°. Concentration of excess phosphotungstic acid, approximately 50 gm. per liter. Concentration of HCl, 0.25 N.

Proportions of each amino acid in pptd. mixtures		Hydroxylysine nitrogen found in 1 liter filtrate
Hydroxylysine	Lysine	
<i>per cent</i>	<i>per cent</i>	<i>mg.</i>
80	20	15
40	60	9
10	90	3

or 0.47 mg. per liter of filtrate, corresponding to 0.94 mg. of hydroxylysine nitrogen per liter.

The analyses of the filtrates, corrected for the blank, indicated the amounts of hydroxylysine remaining in solution per liter of filtrate shown in Table VIII.

*Recovery of Hydroxylysine Added to Proteins*

Horse hemoglobin and gelatin from pig skin were analyzed for hydroxylysine with and without the addition of this amino acid. Of each protein two portions of 3 gm. each were analyzed, and 2.5 mg. of hydroxylysine nitrogen in the form of hydroxylysine picrate were added to one portion of each pair before hydrolysis. The small amount of hydroxylysine was used because it is of the order of magnitude, about 0.5 per cent of the protein nitrogen, in which



hydroxylysine is usually found to occur in proteins. Of the two proteins, hemoglobin represents one entirely lacking hydroxylysine, while the gelatins are the richest in hydroxylysine of any of the proteins yet analyzed. The results are given in Table IX.

The recovery of the added hydroxylysine from the diamino acid fraction of the hemoglobin was 87 per cent of that added; from the gelatin it was 76 per cent. The losses, attributable to solubility of lysine phosphotungstate in the mixed precipitate of the diamino

TABLE IX  
*Recovery of Hydroxylysine Added to Proteins before Hydrolysis*

Protein analyzed	Hydroxy- lysine N added	Total nitrogen of sample hydrolyzed	Hydroxy- lysine N of diamino acid fraction*	Hydroxyamino N of filtrate from phospho- tungstate ppt.	
				Filtrate from 1st ppt.	Mother liquors from recrystallized ppt.
	mg.	mg.	mg.	mg.	mg.
Hemoglobin (horse)	0	456.6	0	33.24	0.22
Hb + hydroxylysine	2.5	459.1	2.19	33.80	0.28
Increase due to added hydroxylysine		2.5	2.19	0.56	0.06
Gelatin from pig skin	0	484.7	3.59	19.10	0.29
" + hydroxylysine	2.5	487.2	5.50	19.46	0.38
Increase due to added hydroxylysine		2.5	1.91	0.36	0.09

\* Calculated as twice the hydroxyamino nitrogen determined as ammonia.

acids, have been discussed under "Error due to solubility" in connection with the description of the method.

The quantitative efficiency of the washing and recrystallization in removing hydroxy acids of the monoamino fraction from the phosphotungstate precipitate may be estimated from the analyses of the mother liquors of the recrystallized phosphotungstates in the last two columns of Table IX. In the case of hemoglobin, which may be accepted as entirely free of hydroxylysine, the filtrate from the first precipitation contained 33.24 mg. of hydroxyamino nitrogen of the monoamino acid group. Recrystallization of the precipitate yielded a mother liquor with only 0.22 mg. of

such nitrogen, or 0.3 per cent as much, and only 0.05 per cent of the total protein nitrogen. It would seem that each crystallization and washing of the phosphotungstate removed about 99.7 per cent of the serine and other hydroxy acids of the monoamino fraction, and that the two precipitations should in general assure leaving an amount in the precipitate equal to less than 0.01 per cent of the total protein nitrogen.

TABLE X  
*Hydroxylysine Content of Proteins*

Protein analyzed	Hydroxylysine N in protein hydrolysate
	<i>per cent of total N</i>
Horse hemoglobin.....	0
Arachin.....	0.01
Lactoglobulin.....	0.02
Lactalbumin.....	0.03
Mixed proteins of horse plasma.....	0.03
Crude papain A <sub>2</sub> .....	0.04
Egg albumin scales.....	0.09
Watermelon seed globulin.....	0.10
Wool.....	0.11
Gliadin.....	0.12
Cottonseed globulin.....	0.23
Casein.....	0.33
Zein.....	0.33
Aleuronate.....	0.54
Gelatin from pig skin.....	0.73
Collagen from cattle Achilles tendon.....	0.88
Gelatin, Coignet, gold label.....	0.89
"    "neutral".....	0.90

*Hydroxylysine Determination in a Series of Proteins<sup>5</sup>*

The results in Table X were obtained by the method described in this paper, with manometric determination of the ammonia formed by periodate acting on the diamino acids.

<sup>5</sup> For the preparations of casein, "neutral gelatin," zein, watermelon seed globulin, cottonseed globulin, and arachin, we thank Dr. H. B. Vickery. For the preparations of crystalline horse hemoglobin, gelatin from pig skin, collagen, lactoglobulin, lactalbumin, and papain we thank Dr. M. Bergmann. For the preparation of aleuronate we thank Dr. Charles L. Hoagland.

No correction has been attempted for the solubility of hydroxylysine phosphotungstate, because, as pointed out, the solubility decreases with the proportion of hydroxylysine in the diamino acid mixture. Because of this behavior it appears that if even minute amounts of hydroxylysine are present they will be carried down with the precipitate. Hence the fact that in ten of the sixteen proteins analyzed hydroxylysine nitrogen amounted to not over 0.1 per cent of the total nitrogen must be accepted as evidence that these proteins contained very little of this amino acid.

Error in the opposite direction, results too high because of occlusion of serine and threonine in the phosphotungstate precipitate, was estimated from the analyses of the mother liquors in Table IX to be probably not over 0.01 per cent of the total nitrogen.

The most striking thing about the hydroxylysine contents is the fact that in no protein does the hydroxylysine nitrogen amount to more than 1 per cent of the total nitrogen.

Collagen and gelatin are characterized by higher hydroxylysine contents than any of the other types of proteins analyzed. The amount in gelatin, 0.9 per cent of the total nitrogen, would approximate 1.0 per cent if corrected for solubility of the phosphotungstate in the mixture precipitated. It is about 3 times as much as we have isolated from this protein in the form of picrate by the incomplete methods of separation that have been available.

#### SUMMARY

Hydroxylysine in protein hydrolysates has been determined by precipitating it with the other diamino acids as phosphotungstate, and determining the ammonia evolved from the  $\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot$  group of the hydroxylysine when the diamino acids are treated with alkaline periodate.

Under the conditions employed for the periodate-ammonia reaction with hydroxylysine, the other amino acids with the  $\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}(\text{OH})\cdot$  group, *viz.* serine, threonine, and  $\beta$ -hydroxyglutamic acid, also give quantitative yields of ammonia; no amino acid without this group has been found to yield ammonia. In the analysis of protein hydrolysates for hydroxylysine, the other ammonia-yielding amino acids are completely separated from the hydroxylysine by recrystallization of the diamino acid phosphotungstates.

In a series of sixteen proteins analyzed for hydroxylysine only six were found in which hydroxylysine contained over 0.1 per cent of the total nitrogen, and in only gelatin and collagen did the amount approach 1 per cent of the total protein nitrogen.

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## LETTERS TO THE EDITORS

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### THE FORMATION OF LANTHIONINE ON TREATMENT OF INSULIN WITH DILUTE ALKALI

*Sirs:*

The fate of the sulfur of insulin during the inactivation of insulin by dilute alkali has long been a controversial subject. Whether or not the inactivation of the insulin is due solely to the effect of the alkali upon the dithio linkages is yet an open question.<sup>1</sup> However, during alkali inactivation of insulin there is destruction of cystine<sup>2,3</sup> and a liberation of sulfide sulfur.<sup>2,4</sup> However, no experiments have as yet been performed which account for all of the cystine sulfur which disappears under dilute alkali treatment.

The isolation of the new sulfur-containing amino acid, lanthionine, by Horn, Jones, and Ringel<sup>5</sup> and Horn and Jones<sup>6</sup> from wool, human hair, and lactalbumin which had first been treated with dilute sodium carbonate suggested to us that the formation of lanthionine might explain, at least in part, the fate of the cystine sulfur unaccounted for in alkali-treated insulin. This possibility was therefore investigated with the following results.

When 2.95 gm. of amorphous insulin (letin powder W-1002, 21 to 22 units per mg.)<sup>7</sup> were treated with 40 cc. of 2 per cent

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<sup>5</sup> Horn, M. J., Jones, D. B., and Ringel, S. J., *J. Biol. Chem.*, **138**, 141 (1941).

<sup>6</sup> Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, **139**, 473 (1941).

<sup>7</sup> The authors wish to thank Eli Lilly and Company for the insulin sample and for the research grant which has aided this work.

sodium carbonate at 100° for 1 hour, 88 per cent of the cystine, as determined by the Sullivan<sup>8</sup> method, was destroyed. The sample was then hydrolyzed with HCl and lanthionine was isolated by the procedure of Horn, Jones, and Ringel.<sup>5</sup> 29 mg. of the typical triangle-like crystals of mesolanthionine were isolated.<sup>9,10</sup> This represents a yield of 5.5 per cent of the total sulfur of the insulin as mesolanthionine and is of the same order of magnitude as the yield obtained from wool. This sample contained 15.16 per cent sulfur (theoretical value 15.40). The benzoyl derivative melted at 208° (corrected), and when mixed with an authentic sample, showed no depression of the melting point.

It was found that lanthionine was also formed by the action of N/30 NaOH on insulin. A 500 mg. sample was treated with 8 cc. of N/30 NaOH at 38° for 96 hours, at which time 92 per cent of the cystine was found to have been destroyed. This sample was then hydrolyzed and about 5 mg. of the typical mesolanthionine crystals were isolated.

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<sup>8</sup> Sullivan, M. X., and Hess, W. C., *Pub. Health Rep., U. S. P. H. S.*, suppl. 86 (1930).

<sup>9</sup> du Vigneaud, V., and Brown, G. B., *J. Biol. Chem.*, **138**, 151 (1941).

<sup>10</sup> Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **140**, 767 (1941)

## STEROIDS

### V. $\alpha$ -ESTRADIOL AND PROGESTERONE METABOLISM\*

*Sirs:*

To gain insight into the course and interrelationship of the metabolism of the estrogenic and progestational hormones, a chemical study has been made of the fate in the rabbit of  $\alpha$ -estradiol and progesterone injected separately and concomitantly.

In all experiments, 300 mg. of each or either hormone were administered over 7 days to a pair of adult females. The urine was extracted with ether after  $\frac{1}{2}$  hour in the autoclave at  $121^\circ$  and pH 1.8, and the ether-soluble material was divided in the usual way into acidic, ketonic and non-ketonic phenolic, and neutral alcoholic fractions, from which the respective metabolites were obtained in crystalline state by adsorption on alumina and fractional elution with solvent mixtures. All products were identified by mixed melting point determinations and as derivatives.

In the intact estrous animal, 300 mg. of  $\alpha$ -estradiol gave 7.8 mg. (2.6 per cent) of estrone (m.p.  $248-252^\circ$ ) and 36.2 mg. (12.1 per cent) of  $\beta$ -estradiol<sup>1</sup> (m.p.  $222-224^\circ$ , m.p. of diacetate  $140-142^\circ$ ). The simultaneous administration of progesterone and  $\alpha$ -estradiol or estrone to the intact estrous (three experiments) and to the hysterectomized-ovariectomized (one experiment) rabbit did not alter the qualitative picture; in all cases  $\beta$ -estradiol and estrone were obtained in the proportion of 4-5 to 1. Chromatographic separation of the non-ketonic phenols in each instance into 40 to 80 fractions with an average weight of 1 to 2 mg. yielded no estriol, nor could its presence in any of these fractions be demonstrated by the David color reaction. That the conditions of

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<sup>1</sup> See also Stroud, S. W., *J. Endocrinology*, **1**, 201 (1939), and Fish, W. R., and Dorfman, R. I., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. xl (1941).



processing of the urine permit of the isolation of estriol was shown by the recovery of 29.6 mg. (m.p. 276–280°) after injection of 300 mg. Thus the chemical findings fail to substantiate the hypothesis that estriol formation from  $\alpha$ -estradiol or estrone takes place in the uterus under the influence of progesterone, a concept elaborated by colorimetric and bioassay of the “weak” and “strong” phenols of rabbit and human urine.<sup>2</sup>

Pregnane-3( $\alpha$ ), 20( $\alpha$ )-diol is the chief metabolite of progesterone in the rabbit as in the human, and the extent of the conversion is about the same. The diol (m.p. 238–240°, m.p. of diacetate 178–180°) was isolated from the neutral alcohols of the urine of all progesterone-treated animals in quantity equivalent to 7 to 11 per cent of the luteoid given; no other reduction product was encountered. It is not, however, eliminated in conjugation with glucuronic acid, as evidenced by failure to obtain pregnanediol glucuronide from unhydrolyzed urine on application of the Venning<sup>3</sup> method. Almost as much pregnanediol was recovered in the absence of the uterus, which bears out clinical observations<sup>4</sup> and suggests that the reduction is more probably concerned with the inactivation of the hormone than with its utilization in causing progestational proliferation.

The investigations are being extended; full details will be published shortly.

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# A MICROMETHOD FOR THE DETERMINATION OF ARGININE

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Micromethods for the determination of arginine based on the use of the Sakaguchi reagent have been described (1-4). This reagent gives a strong color with glycoyamine, arginine, and other monosubstituted guanidine derivatives.

In a previous communication (5) a method for the determination of glycoyamine was described based on the Sakaguchi reaction and the quantitative separation of glycoyamine from arginine by selective adsorption of the arginine on permutit. In the method outlined below the separated arginine is eluted from the permutit and determined independently.

A number of common non-chromogenic substances such as ammoniak, urea, histidine, and creatine reduce the color obtained with the Sakaguchi reagent. A change in the usual order of addition of the color reagents similar to that previously described (5) decreases the interference by these compounds.

## *Reagents and Apparatus—*

3 per cent sodium chloride.

0.3 per cent sodium chloride.

Permutit according to Folin. Permutit can be regenerated after use by allowing 3 per cent sodium chloride to percolate through and then washing with distilled water until chloride-free. This is most conveniently done in large batches on a Buchner funnel.

10 per cent urea in water.

0.2 per cent naphthol in absolute alcohol, diluted with 4 volumes of the 10 per cent urea solution before use.

Hypobromite solution. 0.66 ml. of liquid bromine are added

to 100 ml. of 5 per cent sodium hydroxide. Since this solution deteriorates, it should not be kept for more than 1 or 2 days, and is best kept in a refrigerator.

10 mg. per cent arginine in 0.1 N hydrochloric acid. Standard solutions are made on the day on which they are to be used, by diluting this stock solution with water.

Adsorption column. The permutit is contained in the stem of a glass funnel whose dimensions are: upper part 15 mm. external diameter, 100 mm. long; stem 7 mm. external diameter, 100 mm. long. The lower end of the stem is slightly constricted. A small amount of cotton is placed above the constriction. 0.9 gm. of permutit is poured in and tapped gently to settle the particles.

A 0.2 ml. micro pipette whose contents are delivered under pressure. The delivery time should be sufficiently short to insure rapid delivery and complete mixing (6).

*Preparation of Solutions for Analysis*—For complete separation of glycocyamine and arginine the salt concentration of the solution should not be over 0.5 per cent. If neither of these compounds is present in amounts over 2 mg. per cent, the salt concentration may be as high as 1 per cent. Urine is usually diluted 5 to 10 times with water. Blood filtrates may be prepared by deproteinizing according to Folin and Wu or by heat coagulation at pH 6 after 1:10 dilution with water. Tissue extracts are diluted to contain 1 gm. of tissue (fresh weight) in 40 ml. of suspension. The pH is adjusted to 6.0, and the suspension immersed in a boiling water bath for 10 minutes, cooled, and filtered. Analyses are carried out on the filtrates.

#### *Procedure*

5 ml. of the solution to be analyzed are passed through the permutit column and the small amount of glycocyamine remaining in the column is removed with 5 ml. of 0.3 per cent sodium chloride. The combined filtrate contains all the glycocyamine. A test-tube graduated at 10 ml. is now placed under the funnel, and the arginine is eluted by passing 10 ml. of 3 per cent sodium chloride through the column. The solution in the test-tube is made up to the 10 ml. mark and shaken.

A 2 ml. aliquot is taken for analysis. It is first cooled in an ice bath, then 0.5 ml. of the ice-cold naphthol-urea solution is added,

and after 2 minutes 0.2 ml. of ice-cold sodium hypobromite solution added by means of the micro pipette. This pipette is placed above the solution level and kept away from the sides of the test-tube to prevent contamination by urea in the solution. The color is simultaneously developed in a series of standard solutions containing 0, 0.25, 0.5, 1.0, and 2.0 mg. per cent of arginine.<sup>1</sup> After 20 minutes the color development is complete and remains stable for 2 hours if the solutions are kept in an ice bath. The tubes are shaken for a few seconds to remove excess gas, warmed by immersion in water at room temperature, and the intensity of

TABLE I  
*Elution of Arginine from Permutit Column*

5 ml. of a 2 mg. per cent solution of arginine in 0.5 per cent sodium chloride were passed through the adsorption column followed by 5 ml. of 0.5 per cent sodium chloride. The arginine was then eluted by the salt solution as shown.

Sodium chloride eluting solution		Arginine recovered
Amount	Concentration	
ml.	per cent	
10	0.5	0
5	1.0	2
10	1.0	40
5	2.0	60
10	2.0	100
10	3.0	100
10	5.0	100
5	5.0	88
10	10.0	88

the color measured in a spectrophotometer or a colorimeter, with light of approximately  $0.525 \mu$  (yellow-green).

#### DISCUSSION

As shown in Table I, the complete removal of arginine from the permutit column depends on the amount as well as the concentration of salt solution used. Under the conditions described, the separation of glycocyamine and arginine is complete over a wide

<sup>1</sup> If an appropriate correction factor is applied, glycocyamine standards which are stable may be used.

range of concentrations (Table II). With concentrations of arginine of over 2 mg. per cent the color is too intense to be read and must be diluted before the color is developed. The blanks are yellow instead of colorless as in the procedures described in the literature.

In these older procedures a large excess of urea is added after a given interval to prevent destruction of the chromogenic compound by the hypobromite. During this interval other substances may compete for the hypobromite and reduce or completely inhibit the formation of color. The advantage of the procedure described

TABLE II

*Limiting Concentrations of Glycocyamine and of Arginine That Can Be Separated on Permutit Column*

All values are concentrations in mg. per cent. The concentrations were those of the solutions passed through the permutit column. The final filtrates were diluted to bring the concentration to approximately 1 mg. per cent before the color was developed.

Arginine added	Glycocyamine added	Arginine found
0.0	2.0	0.0
0.0	2.5	0.0
0.0	5.0	0.0
0.0	10.0	0.0
1.0	0.0	1.0
2.0	0.0	2.0
5.0	0.0	5.0
10.0	0.0	9.6
20.0	0.0	18.4

above is that this interference is greatly reduced and for most purposes is negligible.

Table III shows the recovery of arginine added to urine, kidney extract, and blood.

Weber (2) has studied the influence of some non-chromogenic substances on color development. He found that 6 mg. per cent of ammonia, 12 mg. per cent of histidine dihydrochloride, 6 mg. per cent of tyrosine, 6 mg. per cent of tryptophane, 40 mg. per cent of creatine, or 160 mg. per cent of urea<sup>2</sup> "either prevents all color

<sup>2</sup> These are the concentrations in the final diluted filtrates.

formation or the color is so altered that even for qualitative purposes the test is worthless." With the method described in this communication, 60 mg. per cent of ammonia, 5 mg. per cent of histidine hydrochloride, 8 mg. per cent of tyrosine, 8 mg. per cent of tryptophane, 20 mg. per cent of creatine, and 2000 mg. per cent of urea are without influence on the recovery of arginine. If tryptophane and histidine are present in excess of the

TABLE III

*Recovery of Arginine Added to Urine, Kidney Extract, and Blood*

All the values are concentrations in the eluate measured in mg. per cent. The urine was diluted 1:4 with water. The kidney extract was made by grinding up 1 part by weight of kidney with 40 parts of 0.5 per cent sodium chloride, bringing the extract to pH 5.0, boiling 10 minutes, cooling, and filtering. The analysis was carried out on the protein-free filtrate. In both the kidney extract and blood, arginine was added before protein precipitation.

Arginine added	Arginine					
	Urine		Kidney extract		Folin-Wu blood filtrate	
	Found	After subtraction of blank value	Found	After subtraction of blank value	Found	After subtraction of blank value
0.0	0.13		0.15		0.19	
	0.13		0.14		0.20	
0.2	0.345	0.215			0.41	0.21
	0.34	0.210			0.43	0.22
0.5	0.61	0.48	0.65	0.505	0.68	0.48
	0.61	0.48	0.655	0.51	0.68	0.48
1.0	1.12	0.99	1.19	1.045	1.19	0.99
	1.13	1.00	1.19	1.045	1.19	0.99
1.5			1.64	1.50	1.67	1.47
			1.64	1.50	1.71	1.51

amount given, they are chromogenic, and, a further complication, histidine reduces the color given by the arginine present. In pregnancy urine the concentration of histidine may be as high as 160 mg. per cent (7) and the method is inapplicable as it stands. The method is satisfactory for normal urine, although the possible presence of other chromogens, such as methylguanidine, must be borne in mind.

## SUMMARY

A micromethod for the determination of arginine in biological fluids and tissue extracts is described. The advantages of this new method are that a complete separation of arginine from glycocyamine is effected, and that it gives satisfactory results in the presence of common biological substances which interfere in the procedure of previous methods.

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## THE CONVERSION OF CITRULLINE TO ARGININE IN KIDNEY\*

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Glycocycamine is formed in the kidney by the transfer of the amidine group of arginine to the nitrogen atom of glycine. In the study of this reaction it was observed that glycocycamine was also formed from citrulline and glycine. No other donor or precursor of the amidine group was found (1).

The most probable explanation of this effect of citrulline was that it was first converted to arginine, from which the amidine group was then transferred to glycine. This has now been proved.

This formation of arginine consists in an interaction of citrulline with either glutamic acid or aspartic acid. Its speed is of the same order of magnitude as that of oxidative deamination in the kidney. The two dicarboxylicamino acids are equally effective in this respect. An oxidation is involved in the reaction; it is nearly completely inhibited by such oxidation inhibitors as KCN,  $\text{As}_2\text{O}_3$ , and  $\text{As}_2\text{O}_5$  in low concentration. The KCN inhibition is partly relieved by hydrogen acceptors; that of  $\text{As}_2\text{O}_3$  and  $\text{As}_2\text{O}_5$  is not.

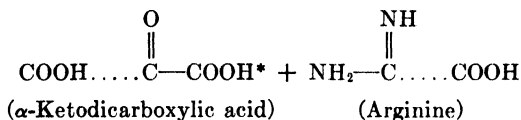
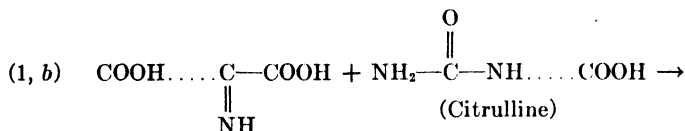
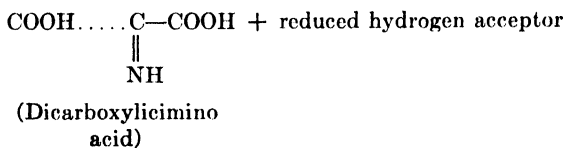
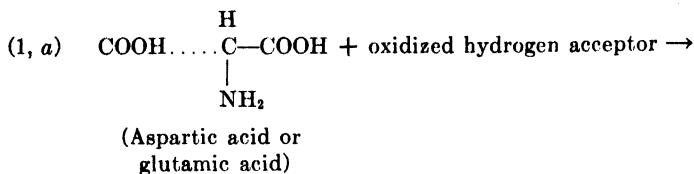
There are in general three possible types of mechanism for this reaction, the essential differences in them consisting in the point at which the oxidation, *i.e.* the dehydrogenation, occurs: (a) at the dicarboxylicamino acid before it reacts with the citrulline, (b) at an intermediate compound consisting of the citrulline and the dicarboxylicamino acid, or (c), after the cleavage of this hypothetical intermediate compound, at a derivative of the citrulline which is the immediate precursor of the arginine.

\* Aided by a grant from Mr. I. Zellerbach.

A summary of this work has appeared (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **140**, p. xviii (1941)).



The first of the three possibilities is depicted by the following equations,



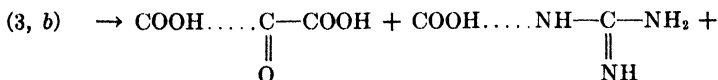
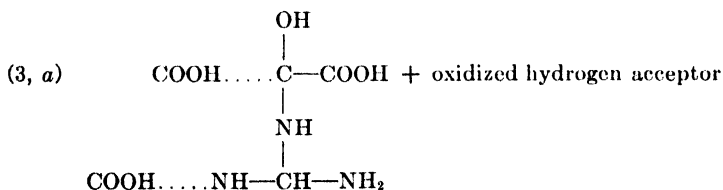
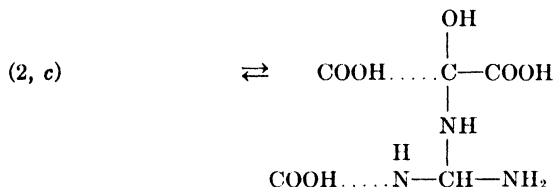
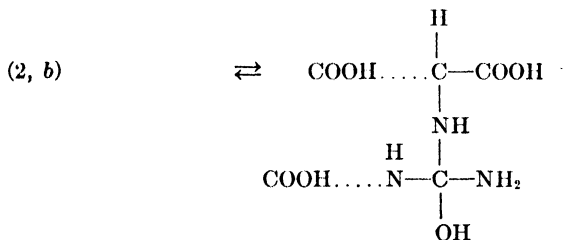
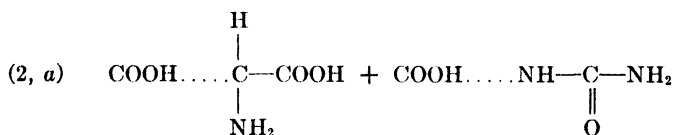
\* Here, as in Equations 2 to 4, the dicarboxylic acid is written as being converted to the corresponding keto acid derivative. This is done only for convenience. We have obtained no evidence regarding the nature of the product arising from the deaminized dicarboxylicamino acid. It is possible that it is also decarboxylated in the oxidation; *i.e.*, the reaction mechanism is that suggested by Herbst (2).

This mechanism was eliminated by the finding that arginine formation is inhibited under conditions in which oxidative deamination of the dicarboxylicamino acids proceeds unchecked.

Arginine is also formed from citrulline, ammonia, and α-keto-glutarate or oxalacetate. These reactions are also inhibited by KCN or As<sub>2</sub>O<sub>3</sub>. The interpretation we have placed on these results is that here also the reaction proper (*i.e.*, arginine formation) is between citrulline and glutamic acid or aspartic acid; that the dicarboxylicamino acids were formed by reductive amination prior to their combination with citrulline. It follows that the imino

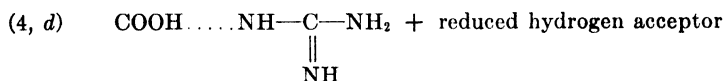
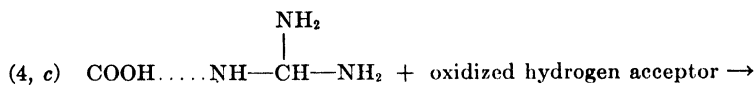
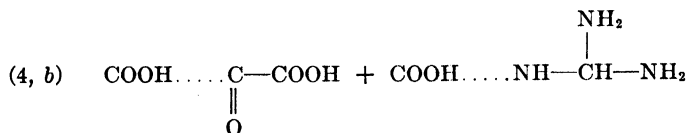
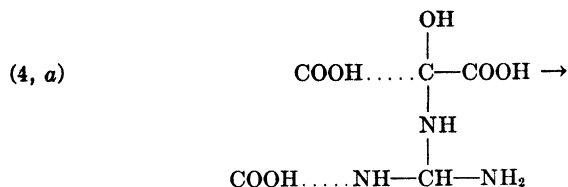
acid derivatives of the dicarboxylicamino acids (which are formed spontaneously and are in equilibrium in water with their corresponding  $\alpha$ -keto acids and ammonia) do not react with the citrulline. This evidence also therefore excludes the reaction mechanism of Equations 1, *a* and 1, *b*.

The other two possible mechanisms are given by Equations 2, 3, and 4.



reduced hydrogen acceptor

or



In both mechanisms an intermediate compound between the citrulline and the dicarboxylic acid is postulated. According to the mechanism of Equations 3, *a* and 3, *b* this compound is decomposed by dehydrogenation into arginine and the  $\alpha$ -keto acid derivative of the dicarboxylicamino acid.

In the mechanism of Equations 4, *a* to 4, *d* the hypothetical intermediate compound is decomposed first and the resulting diamine group undergoes dehydrogenation to form arginine.

If the mechanism of Equations 4, *a* to 4, *d* were correct, when citrulline and the dicarboxylicamino acid are present but arginine formation is prevented by  $\text{As}_2\text{O}_3$ , the keto acid should be formed nevertheless, but without an equivalent amount of ammonia. Ammonia and keto acid will be formed in addition from the concurrent and independent oxidative deamination of the dicarboxylicamino acid. Accordingly the ratio  $\Delta \text{NH}_3$  to  $\Delta$  keto acid should be lower and the absolute amount of keto acid higher in solutions containing citrulline, dicarboxylicamino acid, and  $\text{As}_2\text{O}_3$  than in the absence of citrulline; *i.e.*, the reaction would proceed as far as Equation 4, *b*. It was found that the ratio  $\Delta \text{NH}_3$  to  $\Delta$  keto acid in the presence of  $\text{As}_2\text{O}_3$  was the same with and without citrulline; nor was there an absolute increase in keto acid in the presence of citrulline.

The evidence appears to lead by exclusion to a mechanism of the type of Equations 2 and 3. Braunstein (3) postulated an intermediate compound analogous to that of Equation 2 as a preliminary to transamination. It must be emphasized that in both cases the existence of this hypothetical compound is, as yet, unsupported by any direct evidence. It is an inference only.

Positive results were obtained when proline, hydroxyproline, ornithine, or lysine was used in place of aspartic acid or glutamic acid. All the other amino acids were negative. There is independent evidence that the first three amino acids named are readily convertible to glutamic acid in the rat (4-7). It is reasonable to conclude, therefore, that lysine also is converted to glutamic acid (or aspartic acid). This is the first clue, as far as we are aware, regarding the path of degradation of the carbon skeleton of lysine in the animal body.

In the ornithine cycle by which urea is formed in the liver from ammonia and carbon dioxide, one of the steps proposed is the conversion of citrulline to arginine (8). The mechanism for this conversion which we have found in kidney, *i.e.* the interaction of citrulline with aspartic acid or glutamic acid, does not occur in liver. A study of arginine formation in liver is in progress and will be reported later.

#### *Procedure and Preparations Used*

Most of the experiments were carried out with surviving rat kidney slices. In a few experiments guinea pig kidney was used. The details of the technique have been described (9). Two or three small slices were suspended in 4 ml. of bicarbonate-Ringer's solution containing the substances under investigation. The pH of the solution was 7.4 after equilibration with 95 per cent oxygen and 5 per cent CO<sub>2</sub>. All the experiments were carried out at 38° for 1 or 2 hours.

At the end of the experimental period the contents of the reaction vessels, acidified to pH 6.0 with a drop of 0.5 N HCl, the slices, and two 2.5 ml. washings with water were transferred to test-tubes graduated at 10 ml. After 10 minutes in a boiling water bath the test-tubes were cooled, and the contents made up to the mark with water, mixed by shaking, and filtered. The tissue slices were dried at 100° and weighed.

Glycocyanine and arginine were then determined in the protein-free filtrates. A 5 ml. aliquot was passed through a column of permutit and the traces of glycocyanine remaining in the column removed with 5 ml. of 0.3 per cent NaCl. The glycocyanine was determined in the combined filtrate by the method we have described (10). The arginine was then eluted from the permutit and determined as described by Dubnoff.<sup>1</sup>

Ammonia and keto acids were also determined in some experiments. These analyses were carried out on the protein-free filtrates which were not passed through permutit. The analytical method used for ammonia has been described (11).

The keto acids were determined as follows: To 2 ml. of protein-free filtrate 0.3 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 1 N HCl was added, followed after standing for a few minutes by 1 ml. of 1 per cent NaOH. An intense, deep red color appears immediately. This quickly fades and after 20 minutes a stable red color remains whose intensity is proportional to the concentration of pyruvic acid, oxalacetic acid, or  $\alpha$ -ketoglutaric acid present. A linear relationship is obtained between the concentration of keto acid and the depth of color when it is measured at a wave-length of 0.525  $\mu$ , in the concentration range from 0 to 2.0 mg. per cent. The color of higher concentrations is too deep to be measured accurately.

The use of 2,4-dinitrophenylhydrazine in alkaline solution for the colorimetric determination of pyruvic and other keto acids has recently been reviewed by Klein (12). The simple procedure we have employed, without preliminary extraction of the hydrazone, would give positive values with such substances as acetone and acetoacetic acid. Under our experimental conditions the latter two substances are not formed in measurable amounts. The controls (Table VII) showed that for our limited purposes the simplified procedure was satisfactory.

*l*(+)-Citrulline was used throughout. It was prepared by a combination of the methods described by Kurtz (13) and by Gornall and Hunter (14). The starting material was a commercial preparation of *l*(+)-ornithine. All the other amino acids used were prepared or purified either by Amino Acid Manufactures, University of California at Los Angeles, or in this laboratory. We are in-

<sup>1</sup> Dubnoff, J. W., *J. Biol. Chem.*, **141**, 711 (1941).

debted to Professor H. B. Vickery for a generous donation of glutamine. The naturally occurring isomers of the amino acids were used except when the *dl* form is specified.

Pyruvic acid was prepared by distillation of a commercial product; the distillate was neutralized by NaOH, and the sodium salt crystallized and dried.

$\alpha$ -Ketoglutaric acid was prepared by the method of Neuberg and Ringer (15). We are indebted to Mr. Werner Baumgarten for this preparation.

Oxalacetic acid was prepared by the method of Simon (16).

The  $\alpha$ -keto acid derivative of methionine was obtained by oxidation of *dl*-methionine with *d*-amino acid oxidase prepared from hog kidney, ethereal extraction of the acid from the acidified, deproteinized solution, evaporation of the ether, solution of the acid in water, neutralization with NaOH, and recrystallization from water.

The acetaldehyde was a commercial preparation.

Coenzyme I was prepared by the method of Ohlmeyer (17), and coenzyme II and adenosine triphosphate according to the prescription given by Warburg and Christian (18). The coenzyme preparations, as used, were 60 per cent pure. These preparations were made by Dr. Norman H. Horowitz.

### *Results*

Table I is a typical protocol showing the formation of arginine from citrulline and glutamic acid or aspartic acid. There was always a significant increase in arginine over the blank value when citrulline alone was added to the Ringer's solution. This arginine was formed, presumably, from the added citrulline and aspartic acid or glutamic acid (see Table IV) which was present in the kidney slices at the outset or formed in the slices during the experiment. Nearly 3 times as much arginine was formed, however, when either aspartic acid or glutamic acid was added with the citrulline. The two dicarboxylicamino acids were equally effective.

There was a small increase in glycocyamine whenever arginine formation occurred. It may be inferred from previous observations (1) that the amidine group of the arginine was transferred to glycine which was present in the slices initially or formed in the course of the experiment. The quantity of available glycine was

small and also limited because no more glycoeyamine was formed when the arginine was increased 3-fold.

It was not feasible, because of the high solubility of arginine and its salts, to attempt to prove by isolating one of them that the substance we were measuring colorimetrically was arginine. This was

TABLE I

*Typical Protocol Showing Formation of Arginine by Surviving Rat Kidney Slices from Citrulline and Glutamic Acid or Aspartic Acid*

Ringer's solution; 38°; 1 hour.

Solution No. (1)	Tissue weight, dry (2)	Citrulline, 0.0025 M (3)	Glutamic acid, 0.005 M (4)	Aspartic acid, 0.005 M (5)	Arginine found		Glycoeyamine found	
					(6) mg. per cent	(7) mg. per gm. fresh tissue	(8) mg. per cent	(9) mg. per gm. fresh tissue
1	3.2	—	—	—	0.02	0.3	0.02	0.3
2	7.3	—	—	—	0.02	0.1	0.04	0.2
3	7.2	+	—	—	0.33	1.8	0.07	0.4
4	6.7	+	—	—	0.31	1.9	0.07	0.4
5	11.7	—	+	—	0.04	0.1	0.04	0.1
6	11.1	—	+	—	0.04	0.1	0.06	0.2
7	8.3	+	+	—	1.07	5.2	0.08	0.4
8	6.2	+	+	—	0.89	5.7	0.07	0.4
9	10.3	—	—	+	0.03	0.1	0.04	0.2
10	11.9	—	—	+	0.04	0.1	0.04	0.2
11	6.1	+	—	+	0.89	5.8	0.06	0.4
12	7.5	+	—	+	1.02	5.4	0.07	0.4

The figures in Columns 6 and 8 give the concentrations of arginine and of glycoeyamine in the protein-free solutions after they had passed through the columns of permutit. The figures in Columns 7 and 9 are obtained by multiplying those in Columns 6 and 8 respectively by the factors,  $4/100 \times 10/4 \times 2 \times 1000/(5 \times W) = 40/W$ , where  $W$  is the dry weight of tissue in mg. The basis of these factors is as follows: The volume of the original, undiluted Ringer's solution was 4 ml.; it was diluted to 10 ml. for deproteinization; in the course of separation of the glycoeyamine and elution from permutit the aliquot used was diluted with an equal volume of salt solution; the fresh weight of the slices is 5 times the dry weight.

established by three independent pieces of evidence: the specificity of the color reagent under the conditions in which we used it, the disappearance of this chromogenic material when subjected to the action of arginase, and the formation of glycoeyamine in the highly specific transamidation reaction with glycine.

The color reagent, the Sakaguchi reagent, gives a positive test with only three common biological substances. These are glyco-cyamine, histidine, and arginine. Glyco-cyamine is separated quantitatively from arginine and histidine by the adsorption and elution procedure employed. Histidine, however, remains on the permutit and is removed with arginine by the 3 per cent sodium chloride solution used for eluting the arginine from permutit. Histidine has about 1 per cent the chromogenic power of arginine. Even if all the added citrulline had been converted to histidine, most of the color developed would still remain to be accounted for.

Table II presents direct evidence that the material in question was arginine. Digestion with arginase removed nearly all of the chromogenic material designated as arginine. The details of the experimental procedure are given in the foot-note to Table II.

The transamidation reaction (*i.e.*, formation of glyco-cyamine from arginine and glycine) is a highly specific test for arginine when an extract of kidney (instead of kidney slices) is used to provide the enzyme. The arginine formed from any citrulline present is negligible for the purposes of this test. The test consists in incubating the material in question with an excess of glycine in the presence of the enzyme. A positive result is indicated by the formation of glyco-cyamine, and the rate of formation of glyco-cyamine is proportional to the initial concentration of arginine.

Table III shows that this criterion also afforded clear evidence that we were dealing with arginine. If the chromogenic substance in experiments such as those in Table I was arginine, it was to be expected that there would be (*a*) little or no glyco-cyamine in Solutions 1, 3, and 4 of Table III, (*b*) large increases in Solutions 2, 5, and 6, with 2 to 3 times as much in Solutions 5 and 6 as in Solution 2, and (*c*) less arginine remaining in this experiment than in the experiment of Table I because of the conversion of some of the arginine to glyco-cyamine; the "total"<sup>2</sup> arginine formed per hour would, however, be the same in Solutions 5 and 6 of Table III as in Solutions 7 and 8 and 11 and 12 of Table I. The results obtained were in complete accord with these predictions.

Table IV summarizes our findings on the relative effectiveness of

<sup>2</sup> The "total" arginine is the sum of the arginine found and of the amount of glyco-cyamine multiplied by 1.49 (the ratio of the molecular weight of arginine to that of glyco-cyamine).



different amino acids and of some other nitrogen compounds as contributors of the  $\text{=NH}$  group in the conversion of citrulline to arginine. Glutamine was the only compound which was as effective as aspartic acid or glutamic acid. This may be taken to indicate the rapid hydrolysis of the added glutamine rather than that the amide itself is effective. An active glutaminase is present in

TABLE II

*Proof from Action of Arginase That Arginine Was Main Chromogen Measured by Colorimetric Method Employed*

The arginine values are measured in mg. per gm. of fresh tissue.

Citrulline, 0.0025 M	Aspartic acid, 0.005 M	Glutamic acid, 0.005 M	Arginine present measured colorimetrically	
			Before action of arginase	After action of arginase
—	—	—	1.4	0.2
+	—	—	3.3	0.2
+	+	—	11.0	0.6
+	—	+	8.8	0.5
—	+	—	1.2	0.2
—	—	+	1.0	0.2

Ringer's solution,  $38^{\circ}$ ; 1.5 hours. Each of the six mixtures was carried through in quadruplicate to the end of the period of incubation with kidney slices; pairs of similar solutions were then combined. Each of the values given above therefore is an average of two completely separate but identical experiments run simultaneously from the initial incubation with kidney slices through the digestion with arginase to the final colorimetric determination. The arginine was determined before and after digestion of the protein-free filtrates with arginase. Dry arginase powder was prepared by the method of Hunter and Dauphinee (19). 4 ml. of the protein-free filtrate were digested at pH 8.7 for 1 hour at  $38^{\circ}$  with 0.2 ml. of a 3 per cent solution of the arginase powder. At the end of the hour the solutions were acidified to pH 6.0, boiled, and filtered. The arginine in the filtrates was then determined in the usual manner after removal of the glycocyamine with permittit.

kidney (20). Asparagine was much less active than aspartic acid (the asparaginase activity of kidney is very low), while acetamide, formamide, and nicotinamide were negative.

The positive results with proline, hydroxyproline, and ornithine were to be expected. Weil-Malherbe and Krebs (4) and Neber (5) have proved that proline and hydroxyproline are converted to

glutamic acid by rat kidney slices. The figures in Table IV show that the formation of arginine from citrulline was approximately twice as fast with proline as with hydroxyproline. This is in accord with the findings of Weil-Malherbe and Krebs that glutamic acid is formed twice as quickly from proline as from hydroxyproline.

The conversion of *l*(+)-ornithine to glutamic acid was first suggested by Krebs (6) on the basis of indirect evidence. *d*(+)-Proline and *d*(-)-ornithine gave the same oxidation product,  $\alpha$ -keto- $\delta$ -aminovalerianic acid, after treatment with *d*-amino

TABLE III

*Glycine Transamidation with Arginine Formed from Citrulline*

The results are expressed as mg. per gm. of fresh tissue.

Solution No.	Metabolites used	Glycocyamine	Arginine	"Total" arginine
1		0	0.4	0.4
2	Citrulline	1.2	2.0	3.8
3	Aspartic acid	0.2	0.8	1.1
4	Glutamic "	0.2	0.6	0.9
5	Citrulline + aspartic acid	2.6	5.9	9.8
6	" + glutamic "	2.7	6.6	10.6

The different mixtures of metabolites were first incubated at 38° with rat kidney slices for 2 hours. The citrulline, aspartic acid, and glutamic acid were initially 0.01 M. A 3 ml. aliquot of each mixture was then removed and added to 1 ml. of a cell-free extract of rat kidney to which glycine had been added to a concentration of 0.04 M. These mixtures were then incubated at 38° with shaking for 6 hours. They were then deproteinized and analyzed for glycocyamine and arginine.

acid oxidase. *l*(-)-Proline was proved to be converted to glutamic acid. It was a reasonable surmise then that glutamic acid was formed also from *l*(+)-ornithine via  $\alpha$ -keto- $\delta$ -aminovalerianic acid in the same manner; *viz.*, oxidation at the  $\delta$ -carbon atom to form the acid and amination of the  $\alpha$ -carbon atom. Direct evidence of this conversion was obtained by Roloff, Ratner, and Schoenheimer (7). These workers fed deuterio ornithine to normal adult mice and later found not only deuterio arginine but also deuterio proline and deuterio glutamic acid. Data such as those in Table IV reveal that the conversion of *l*(+)-ornithine to glutamic acid occurs rapidly in rat kidney.

In view of the findings with proline, hydroxyproline, and ornithine it is a reasonable interpretation of the positive result with lysine (Table IV) that this amino acid also is converted to glutamic acid.<sup>3</sup>

TABLE IV

*Relative Effectiveness of Different Amino Acids and Other Nitrogen Compounds As Contributors of =NH Group in Conversion of Citrulline to Arginine*

Ringer's solution; 38°; 1 hour. The citrulline was initially 0.0025 M; all other compounds 0.005 M. Except where the *dl* form is specified, the naturally occurring isomer was used.

The results are expressed as per cent of "total" arginine formed from citrulline and glutamic acid. "Total" arginine = arginine + glycocyamine  $\times$  1.49.

Substance	Relative speed of arginine formation	Substance	Relative speed of arginine formation
Acetamide	-7	<i>dl</i> -Isoleucine	-8
Alanine	-8	Leucine	-8
Ammonia	1	Lysine	47
Asparagine	55	<i>dl</i> -Methionine	-8
Aspartic acid	108	Nicotinic acid amide	-21
Cysteine	-4	<i>dl</i> -Norleucine	-18
Formamide	-13	Ornithine	42
Glutamic acid	100	<i>dl</i> -Phenylalanine	-12
Glutamine	101	Proline	51
Glutathione	46	<i>dl</i> -Serine	-3
Glycine	1	Threonine	-14
Histidine	0	Tryptophane	-16
Hydroxyproline	23	Tyrosine	-6
		Valine	5

Table V shows the inhibition of the interaction of citrulline and aspartic acid or glutamic acid by low concentrations of KCN, As<sub>2</sub>O<sub>3</sub>, or As<sub>2</sub>O<sub>5</sub>. This inhibition is about 95 per cent complete.

<sup>3</sup> It may be expected from their structural similarity that the conversion of lysine to glutamic acid follows a course analogous to that of ornithine. One pathway for which there is some experimental evidence is as follows: oxidative deamination of the  $\alpha$ -amino group, followed by oxidative deamination of the  $\epsilon$ -amino group,  $\beta$  oxidation at the carbon atom to form  $\alpha$ -ketoglutaric acid, which is reduced with the addition of ammonia to glutamic acid.

The cyanide inhibition is relieved to a considerable extent by the  $\alpha$ -keto acid derivative of methionine, by oxalacetate, and by

TABLE V

*Inhibition of Arginine Formation by Oxidation Inhibitors*

Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of =NH donor 0.005 M.

The results are expressed as mg. of "total" arginine per gm. of fresh tissue.

	Uninhibited	KCN 0.001 M	As <sub>2</sub> O <sub>3</sub> 0.001 M	As <sub>2</sub> O <sub>3</sub> 0.0036 M
No amino acids added.....	1.3	0.9	0.9	0.8
Citrulline.....	2.7	1.1	1.1	1.1
" + aspartic acid.....	12.2	1.8	1.8	1.7
" + glutamic ".....	11.2	1.7	1.8	1.7

TABLE VI

*Counteraction of Hydrogen Acceptors on Inhibition of Arginine Formation by KCN and As<sub>2</sub>O<sub>3</sub>*

Ringer's solution; 38°; 2 hours. The citrulline was initially 0.0025 M; =NH donor and other metabolites 0.005 M.

=NH donor	Aspartic acid			Glutamic acid		
	Uninhibited	0.001 M KCN	0.001 M As <sub>2</sub> O <sub>3</sub>	Uninhibited	0.001 M KCN	0.001 M As <sub>2</sub> O <sub>3</sub>
No amino acids added.....	1.1	0.9	0.9	1.1	0.9	0.9
Citrulline alone.....	3.0	1.0	1.1	3.1	1.0	1.0
" + =NH donor.....	12.2	1.6	1.0	12.0	1.3	1.6
" + " " + acetaldehyde.....		0.9			0.6	
Citrulline + =NH donor + $\alpha$ -keto acid derivative of methi- onine.....		5.5			7.0	
Citrulline + =NH donor + oxal- acetate.....		3.1	1.1		4.3	1.9
Citrulline + =NH donor + pyru- vate.....	7.2	3.1	1.2	6.7	2.2	1.3

pyruvate. These metabolites do not relieve the inhibition by arsenite (Table VI).

Our interpretation of these findings is that both cyanide and

$\text{As}_2\text{O}_3$  (and  $\text{As}_2\text{O}_5$ ) inhibit the formation of arginine by preventing the necessary dehydrogenation of the citrulline-aspartic acid (or glutamic acid) complex. The effect of cyanide is indirect; it prevents the oxidation of cytochrome. The flow of electron and of hydrogen transfer is thus blocked by the lack of an available acceptor. Arginine formation can be reinstituted, however, by the provision of electron and hydrogen acceptors other than the cytochrome-oxygen system which can function even in the presence of cyanide. They must be provided in sufficient concentration because their reoxidation is also blocked by cyanide and they cannot therefore function in a cyclic manner. The  $\alpha$ -keto acid derivative of methionine, oxalacetic acid, and pyruvic acid served this purpose. Acetaldehyde did not relieve the cyanide inhibition. This would exclude complex formation with the cyanide as an explanation of the positive results with the above keto acids.

The arsenite inhibition, on the other hand, is not relieved by these metabolites because this inhibitor interferes with the process of dehydrogenation; *i.e.*, the initial process of donation of electrons and hydrogens is blocked. Hence the provision of additional hydrogen acceptors affords no relief.

In view of the participation of the pyridine coenzymes in the oxidative deamination of *l*(+)-glutamic acid (21) and the evidence that an oxidation of aspartic acid or glutamic acid is involved in this formation of arginine, we tested the effect of coenzymes I and II and of adenylic acid pyrophosphate on the reaction. These substances neither relieved the arsenite inhibition nor increased the rate in the absence of an oxidative inhibitor.

The enzymatic activity of kidney slices is largely lost (about 90 per cent) when the kidney is homogenized into a cell-free suspension. One possible explanation for this loss of activity was that the pyridine coenzymes were dissociated from the protein of the enzyme as a result of the dilution consequent on the preparation of the cell-free suspension. If this explanation were correct, addition of one of the two pyridine coenzymes or of adenylic acid pyrophosphate might be expected to restore some of the lost enzymatic activity. This was not the case. It is possible that some other coenzyme is operative in this reaction.

Another piece of evidence against the above explanation of the loss of enzymatic activity is that kidney tissue homogenized and

tested without any added fluid is no more active than when homogenized and suspended in 4 times its volume of buffer solution.

Table VII shows the separation of oxidative deamination of the dicarboxylicamino acids from the process in which citrulline is converted to arginine. The latter reaction is inhibited by arsenite; the former is not. In fact an inhibitor such as  $\text{As}_2\text{O}_3$  which retards the disappearance of the products of deamination, the ammonia and keto acids, is necessary for the demonstration of oxidative deamination by tissue slices.

TABLE VII

*Separation of Conversion of Citrulline to Arginine from Oxidative Deamination*

Guinea pig kidney slices were used; Ringer's solution;  $38^\circ$ ; 2 hours. The citrulline was initially 0.0025 M; aspartic acid either 0.005 or 0.05 M as indicated;  $\text{As}_2\text{O}_3$  0.001 M.

The results are expressed as micromoles per gm. of fresh tissue.

Citrulline	Aspartic acid, initial molality	$\text{As}_2\text{O}_3$	Arginine found	Keto acid as pyruvic acid	Ammonia	$\frac{\Delta \text{ ammonia}}{\Delta \text{ pyruvic acid}}$
—		—	0.9	0	4.3	
—		+	1.0	0	10.4	
+		—	1.9	0	7.1	
+		+	0.9	0	10.9	
+	0.005	—	12.1	0	8.7	
+	0.05	—	6.3	1.8	13.6	
+	0.005	+	1.7	13.5	25.5	1.6
+	0.05	+	1.3	36.9	54.2	1.4
—	0.005	—	1.0	0	9.0	
—	0.05	—	1.2	0	20.2	
—	0.005	+	1.0	10.4	23.7	1.9
—	0.05	+	1.0	35.1	54.1	1.4

Another piece of evidence which indicated that the two oxidative processes, arginine formation and deamination, are different, although they involve the same substrate (aspartic acid or glutamic acid), was obtained from a comparison of the effects of two different concentrations of aspartic acid, 0.005 and 0.05 M. The higher concentration of aspartic acid inhibited arginine formation, but the rate of deamination, in an absolute sense, was more than 3 times greater. If the reaction consisted in an exchange of the oxygen on the  $\epsilon$ -carbon atom of citrulline for the  $=\text{NH}$  group of

the imino acid derivative of aspartic or glutamic acid, the reaction should proceed with oxalacetic acid and ammonia or ketoglutaric acid and ammonia, even in the presence of cyanide, because the imino acids are formed spontaneously from the latter keto acids and ammonia.

Table VIII shows that arginine is formed from citrulline and the products of deamination of the dicarboxylicamino acids under normal conditions; but the reaction is completely inhibited by cyanide.

TABLE VIII

*Aspartic Acid or Glutamic Acid Formation in Rat Kidney Slices; Attested to by Formation of Arginine from Citrulline*

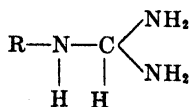
Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of glutamic acid or other metabolites and ammonia 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites in addition to citrulline	"Total" arginine found
None.....	2.4
Citrate + ammonia.....	2.9
Fumarate + ".....	4.8
Glucose + ".....	3.7
Glutamate.....	10.5
$\alpha$ -Ketoglutarate + ammonia.....	7.9
Oxalacetate + ammonia.....	4.8
Pyruvate + ammonia.....	2.6
Succinate + ".....	4.5

Three independent lines of evidence concur, therefore, in excluding prior oxidative deamination of the aspartic acid or glutamic acid as part of the reaction mechanism in the formation of arginine from citrulline.

As stated above, another possible reaction mechanism would locate the oxidation on a derivative of the citrulline after it had undergone transamination with the dicarboxylic acid to form a compound of the type



Dehydrogenation of the diamine group would then yield arginine. If this were the reaction mechanism, the addition of citrulline in the presence of  $\text{As}_2\text{O}_3$  and aspartic acid or glutamic acid would, as discussed above, decrease the ratio  $\Delta$  ammonia to  $\Delta$  keto acid, because of an absolute increase in the keto acids without a corresponding increase in ammonia. The data in Table VII eliminate this hypothesis. In the presence of  $\text{As}_2\text{O}_3$  the absolute amount of keto acids and the ratio of  $\Delta$  ammonia to  $\Delta$  keto acid were the same in the presence as in the absence of citrulline.

The evidence, by exclusion, therefore points to the locus of the oxidation on a hypothetical intermediate compound of citrulline and the dicarboxylicamino acid.

The reason that the ratio  $\Delta$  ammonia to  $\Delta$  pyruvic acid is greater than 1 is that  $\text{As}_2\text{O}_3$  does not completely inhibit the oxidation of keto acids. Even in unpoisoned tissue the removal of ammonia is less complete than that of the keto acids. Similar observations were made by Krebs (6).

The formation of arginine from citrulline by surviving kidney slices can be used as a test for potential sources of aspartic acid or glutamic acid. It was shown by this method that certain amino acids are converted in the kidney to one or the other of these two dicarboxylicamino acids (Table IV). The action of tissue slices in this respect parallels closely that of the whole animal. This parallel was also seen in the synthesis of hippuric acid (22) and of creatine (23). The same method can be used as a test for precursors of the carbon skeleton of the dicarboxylicamino acids. The reagents are citrulline, an excess of ammonia, and the metabolite in question. Some examples are shown in Table VIII. Fumarate, glucose,  $\alpha$ -ketoglutarate, oxalacetate, and succinate were definitely positive. A dubiously slight positive result was obtained with citrate. Pyruvate was negative.

The mechanisms by which these non-nitrogenous metabolites may be converted to the dicarboxylicamino acids are well known and need not be discussed here. The data in Table VIII are positive evidence that these changes occur readily in the kidney. The citric acid cycle appears to be quantitatively less important than simple oxidation of succinic acid and its derivatives.

The formation of arginine may be used as an indicator to ascertain the "preferred" directions of some of the reversible processes



in the dynamic steady state of metabolic reactions in kidney slices. This test is based on the fact that, in the presence of an excess of citrulline, the formation of arginine is faster the greater the concentration of aspartic acid or glutamic acid (at concentrations below 0.005 M). The following are two examples: The amount of arginine formed from citrulline in the presence of different amino acids and  $\alpha$ -ketoglutarate is an indication of the speed of transamination in the direction of glutamic acid formation; the difference in the amount of arginine formed from citrulline,  $\alpha$ -ketoglutarate, and ammonia in the presence and in the absence of

TABLE IX

*Transamination in Rat Kidney Slices*

Ringer's solution; 38°; 2 hours. The initial concentration of citrulline was 0.0025 M; of other amino acids, keto acids, and ammonia 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites in addition to citrulline	"Total" arginine found
None.....	2.0
Glutamate.....	10.9
"    + pyruvate.....	7.8
$\alpha$ -Ketoglutarate.....	3.1
Ammonia.....	2.2
$\alpha$ -Ketoglutarate + ammonia.....	4.6
"    + pyruvate + ammonia.....	1.9
Alanine + $\alpha$ -ketoglutarate.....	2.8
<i>dl</i> -Alanine + $\alpha$ -ketoglutarate.....	2.1
Valine + $\alpha$ -ketoglutarate.....	1.6

pyruvate indicates whether ammonia nitrogen available for amino acid synthesis is bound preferentially as glutamic acid or as alanine.

Table IX is a summary of some experiments along these lines. It is seen that glutamic acid contributes its amino group to pyruvate. On the other hand, amino nitrogen does not pass readily from *l*(+)-alanine, *dl*-alanine, and *l*(+)-valine to  $\alpha$ -ketoglutarate. In this respect ammonia is more effective. We may infer then that the formation of glutamic acid from  $\alpha$ -ketoglutarate is faster by reductive amination than by transamination.

Similarly, in the presence of ammonia,  $\alpha$ -ketoglutarate, and pyruvate there was no evidence of the formation of any glutamic acid available for arginine formation; presumably the nitrogen was bound preferentially as alanine.

The dynamic steady state in kidney slices appears therefore to include a cycle which favors the formation of glutamic acid by reductive amination, followed, if suitable keto acids are available, by transamination to form other amino acids with the regeneration of the  $\alpha$ -ketoglutarate.

TABLE X

*Inhibition by Pyruvate of Arginine Formation from Citrulline*

Ringer's solution; 38°; 1 hour. The initial concentration of citrulline was 0.0025 M; of other amino acids and pyruvate 0.005 M.

The results are expressed as "total" arginine formed per gm. of fresh tissue.

Metabolites added in addition to citrulline	"Total" arginine formed
None.....	1.3
Aspartate.....	5.6
" + pyruvate.....	2.8
Glutamate.....	4.6
" + pyruvate.....	2.3
Lysine.....	3.3
" + pyruvate.....	1.8
Ornithine.....	3.7
" + pyruvate.....	2.4
Proline.....	2.8
" + pyruvate.....	1.1

The situation in kidney slices appears to be similar to that in the whole animal. Schoenheimer and his colleagues (24) observed that the most extensive exchange of normal nitrogen for N<sup>15</sup> occurred in the nitrogen of aspartic acid and glutamic acid whether the N<sup>15</sup> was introduced as ammonia or as a single amino acid.

The formation of arginine from citrulline was inhibited by pyruvate whether the donor of the =NH group was aspartic acid, glutamic acid, or a precursor of these dicarboxylicamino acids (Table X). This is in accord with the findings in Table IX that pyruvate is converted to alanine at the expense of glutamic acid. It is interesting that pyruvate is inhibitory under the normal cir-

cumstances of the experiment of Table X; yet in the presence of cyanide it permits arginine formation to occur, neutralizing to some extent the cyanide inhibition (Table VI). This latter fact suggests strongly that pyruvate does not inhibit the oxidation necessary for the formation of arginine from citrulline, that it is inhibitory under normal circumstances because it competes effectively with the citrulline for the amino group of aspartic acid or glutamic acid. More direct evidence is required, of course, to determine whether or not this hypothesis is correct.

#### DISCUSSION

The work of Braunstein and Kritzmann (3, 25), of von Euler *et al.* (21), and of Cohen (26) established by indirect but strong evidence the central position of the dicarboxylicamino acids in the continual and rapid interchange of amino nitrogen which Schoenheimer and his coworkers demonstrated to occur in the body by direct evidence (24).<sup>4</sup> The mechanism of this interchange involves transamination and reductive amination.

Another mechanism is involved in the formation (in the kidney) of arginine from citrulline in which the dicarboxylicamino acids also participate. Instead of transamination, the reaction might be designated as transimination, except that the dehydrogenation of the amino group of the dicarboxylicamino acid does not occur prior to its reaction with the citrulline but while it is in combination with it.

The discovery of an extremely active mechanism for converting citrulline to arginine in the kidney suggests a hitherto unsuspected source of the arginine which the kidney requires for, among other purposes, the transamidination reaction with glycine to form glycoeyamine.

It seems improbable that a mechanism which can transform citrulline to arginine so quickly and act upon low concentrations of metabolites, less than 0.001 M, is inoperative *in vivo*, teleological as the argument is. The question then arises, what is the source of the citrulline? The kidney cannot convert ornithine to citrulline.

<sup>4</sup> It is interesting in retrospect that this interchange of amino nitrogen could have been inferred from the fact that ammonia exerts considerable nitrogen sparing action even in man. The continual and extensive synthesis and breakdown of protein was deduced from this and other data (27).

If one accepts the ornithine-urea cycle in the liver as proposed by Krebs and Henseleit (8), the liver can hardly supply the citrulline, because in that cycle the existence of the citrulline is only transitory on its way to arginine.

#### SUMMARY

1. Citrulline is converted to arginine at a rapid rate by rat and guinea pig surviving kidney slices. This property is almost completely lost when the cell structure is destroyed.

2. Either aspartic acid or glutamic acid is necessary for this reaction (in addition to citrulline).

3. Proline, hydroxyproline, lysine, and ornithine may replace the dicarboxylicamino acids in this reaction. Evidence is adduced that they do so by being converted first to glutamic acid (or, possibly but less likely, to aspartic acid).

4. Arginine is formed from citrulline and  $\alpha$ -ketoglutaric acid and ammonia or oxalacetic acid and ammonia. Evidence is presented that these products of oxidative deamination are reduced by kidney slices to form the parent dicarboxylicamino acids.

5. This formation of arginine from citrulline is nearly completely inhibited by oxidative inhibitors, KCN,  $\text{As}_2\text{O}_3$ , and  $\text{As}_2\text{O}_5$ , indicating an oxidative step in the reaction mechanism. The cyanide inhibition is relieved in part by the  $\alpha$ -keto acid derivative of methionine, by oxalacetate, and by pyruvate. The inhibition by  $\text{As}_2\text{O}_3$  and  $\text{As}_2\text{O}_5$  is not relieved by these metabolites. An interpretation of these findings is presented.

6. The oxidative step is not a dehydrogenation of the amino group of the dicarboxylicamino acid (to form the imino group) prior to its reaction with citrulline. Evidence is presented that this oxidation may be located at a hypothetical intermediate compound of citrulline and the dicarboxylicamino acid.

7. Arginine formation from citrulline can be used as an indicator of the "preferred" direction of some of the reversible processes in the metabolism of the cells. Some examples are presented.

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# THE EFFECT OF FEEDING *dl*-LYSINE MONOHYDROCHLORIDE ON THE STORAGE OF LIVER GLYCOGEN AND THE URINARY EXCRETION OF ACETONE BODIES\*

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Present knowledge concerning the possible conversion of lysine to glucose or to acetone bodies seems to be based entirely on data obtained by Dakin in two tests on the phlorhizinized dog and two on the surviving liver (1913). Dakin considered the results too indefinite to have demonstrated the formation of either product. Interest in the metabolism of lysine prompted us to reexamine this problem by applying the more recent techniques of determining glycogen deposition in fasting, but otherwise normal, rats after lysine feeding and of measuring the increase or decrease in acetone body excretion after administration of lysine or of glutaric acid (a possible metabolite) either to the fasted rat or to the rat fed sodium butyrate to increase the ketonuria.<sup>1</sup>

## EXPERIMENTAL

The lysine used was the *dl* modification, synthesized as the dihydrochloride by the method of Eck and Marvel (1934) and converted to the monohydrochloride with pyridine.<sup>2</sup> Its purity was verified by analysis for total nitrogen (Scales and Harrison, 1920) and for amino nitrogen (Van Slyke, 1913-14). Although

\* The data presented in this communication are taken from a dissertation submitted in June, 1941, by George O. Sharp in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate College of the State University of Iowa.

<sup>1</sup> A similar study of *dl*-lysine metabolism has appeared since this paper was submitted for publication (Butts and Sinnhuber, 1941). Essentially the same conclusions were reached.

<sup>2</sup> This method of conversion was suggested by Dr. E. T. Mertz. An analogous procedure has since been published (Rice, 1939).

male rats apparently deposit liver glycogen more readily (Deuel, Gulick, Grunewald, and Cutler, 1934), females were used throughout to avoid sex difference in comparisons with studies on acetone body excretion for which the female is preferable (Butts and Deuel, 1933). The rats were secured from Sprague-Dawley, Inc., and prior to the period of fasting (or between fasting periods) were kept on a stock diet of Purina Dog Chow. During the fasting

TABLE I  
*Liver Glycogen Formation from dl-Lysine Monohydrochloride in Female Rats Fasted 48 Hours*

Group No.	No. of rats	Average weight*	Liver glycogen			M.D.† P.E. of M.D.	Total mono-hydro-chloride‡ fed	Additional fasting or feeding period
			Minimum	Maximum	Average			
		gm.	mg. per gm.	mg. per gm.	mg. per gm.		mg. per rat	hrs.
I	6	165	0.20	2.42	0.80		None	0
II	8	170	0.21	2.61	1.15	1.5	532	4
III	8	160	0.23	1.15	0.61	0.82	1064	8
IV	8	167	0.29	1.94	0.89	0.93	1596	12
V	6	171	0.33	1.45	0.64		None	12

\* The weight range was 154 to 181 gm.

† In the calculations of the ratio of mean difference (M.D.) to probable error of mean difference (P.E. of M.D.) control Groups I and V were considered as a single group. Ratios below 3 are usually not regarded as significant.

‡ Feeding of the *dl*-lysine monohydrochloride was by stomach tube in doses of 532 mg. at 4 hour intervals. The solution contained 250 mg. per cc. Analysis of a number of samples indicated that on the average 532 mg. were delivered by the 2 cc. syringe and tube.

periods and the periods of lysine and glutaric acid feeding Cellu flour<sup>3</sup> was available continuously.

*Glycogenesis*—The experimental data for the studies on glycogenesis appear in Table I in condensed form. To secure comparable conditions each rat was injected subcutaneously with 40 mg. of sodium amytal 35 minutes before its liver was removed.

<sup>3</sup> Essentially finely ground sugar-, starch-, fat-, and protein-free vegetable cellulose obtained from the Chicago Dietetic Supply House, Inc. Preliminary tests indicated that its feeding was without effect on glycogen content or on acetone body output.

Of several anesthetics tested in studies involving periodic removal of liver samples over several hours time Murphy and Young (1932) found that sodium amytal caused the least glycogenolysis; we have found its use in similar class projects preferable to stunning. The livers were analyzed in their entirety by an adaptation of the method of Good, Kramer, and Somogyi (1933), in combination with the Somogyi modification (1926) of the Shaffer-Hartmann sugar method. The liver glycogen content of the individuals in each group varied widely, but no more so than in similar analyses (on fasted controls) recorded in the literature. The conclusion that the livers of the experimental groups did not contain appreciably more total glycogen or glycogen per gm. than those of the fasted controls is supported by statistical analysis of the data. Calculations based on the absorption studies of Doty and Eaton (1937-38) suggest that demonstration of glycogen formation should have been possible had even 5 per cent of the glycogen equivalent to 3 of the carbon atoms of the natural *l*(+) component absorbed been deposited in the liver.

*Ketogenesis and Ketolysis (or Antiketogenesis)*—The rats used in the acetone body studies were housed in metabolism cages allowing collection of the urine under oil. The urines were analyzed daily for total nitrogen by the macro-Kjeldahl method (Scales and Harrison, 1920) and for total acetone bodies by the method of Van Slyke (1917). The latter results are recorded as "total acetone bodies calculated as acetone" on the assumption that 75 per cent of the acetone bodies were present as  $\beta$ -hydroxybutyric acid.

The feeding data and averaged analytical results obtained in the two series of tests (Series I and II) on acetone body excretion following the ingestion of *dl*-lysine monohydrochloride are presented in Table II. The considerable variation in daily output of both nitrogen and total acetone bodies in these and the other studies may be attributed in part to biological differences, in part to the failure of the technique to insure the separation of the urine into 24 hour fractions representing the total urine secreted, rather than the urine voided. The output of total acetone bodies by all of the animals was low, but not lower than in some of the studies recorded in the literature. The data on total nitrogen excretion indicate that at least a third of the lysine monohydrochloride fed per day was absorbed. Calculations suggest that even if the *d*(-)-lysine



TABLE II  
Average Total Acetone Body and Total Nitrogen Excretion in Female Rats Fed *dl*-Lysine Monohydrochloride or Glutaric Acid Alone or with Sodium Butyrate after a Preliminary 48 Hour Fast

	Series No. and group	No. of rats*	Total output			Series No. and group	No. rats*	Total output			Days of test	Substances fed (4 equal doses daily)†
			Nitro- gen	Acetone bodies calculated as acetone				Nitro- gen	Acetone bodies calculated as acetone			
				gm. per sq. m.	M. D. P. E. of M. D.				gm. per sq. m.	M. D. P. E. of M. D.		
Lysine monohy- drochlor- ide	I	5	19.09	0.21	1.93	II	5	20.99	0.09	5.06	3	L. (1.63) + NaHCO <sub>3</sub> (0.24)
		4	11.13	0.15			6	11.96	0.05		3	NaCl equivalent to NaHCO <sub>3</sub> above
	III	6	33.76	1.79	1.43	IV	4	29.29	3.76	2.02	4	L. (2.4) + Na butyrate (1.4)
		10	14.80	2.35			4	14.06	7.54		4	" (1.63) + " (1.4)
Glutaric acid	V-A	5	10.80	0.13	9.10	VI-B	5	8.91	0.08	2.83	4	Na butyrate (1.4)
	V-B	5	8.47	0.06		VI-A	5	11.25	0.05		3	G. (0.33) + NaHCO <sub>3</sub> (0.19)
	VII-C	5	9.88	0.33	3.90	VIII-D	5	11.87	0.10	4.46	3	Water (equal volume)
	VII-D	5	9.26	1.18		VIII-C	5	11.33	0.18		4	G. (0.33) + NaHCO <sub>3</sub> (0.19) + Na butyrate (0.275)
												Na butyrate (0.275)
												Na butyrate (0.275)

\* The extreme range in weight of the rats was 190 to 265 gm., the weight distribution about the same in each series. In calculating surface area Mech's formula was used.

† The 0.24 gm. of sodium bicarbonate was equivalent to about one-third of the chloride in the lysine monohydrochloride, the 0.19 gm. not quite half enough to combine with the glutaric acid. The 1.4 gm. of sodium butyrate were equivalent to 2.4 gm. of lysine monohydrochloride and to approximately 22.5 gm. of acetone per sq. m. for rats of the size used; the 0.275 gm. of sodium butyrate was equivalent to the 0.33 gm. of glutaric acid and to approximately 4.4 gm. of acetone per sq. m. L. represents lysine monohydrochloride; G., glutaric acid.

component were completely unmetabolizable, the *l*(+)-lysine would have been ample, if ketogenic, to have caused a small but significant increase in excretion of acetone bodies. Analyses by the acetone body procedure of solutions containing much more lysine monohydrochloride (1.2 gm.) than could have been excreted per day or of sodium chloride (0.6 gm.) equivalent to all of the chloride present in the lysine monohydrochloride fed daily yielded precipitates too small (equivalent to 0.006 gm. of total acetone bodies per sq.m.) to require special consideration. Statistical evaluation of the data indicates that the results obtained are of doubtful significance, particularly in Series I. The data do not warrant concluding that *dl*-lysine monohydrochloride is ketogenic.

Two series of rats (Nos. III and IV) were fed both *dl*-lysine monohydrochloride and sodium butyrate (Table II). In the studies on Series III, which were chronologically the first of all attempted, the 2.4 gm. of lysine monohydrochloride fed each day were equivalent to the sodium butyrate, but because this much lysine monohydrochloride proved to be extremely toxic when fed alone, the dosage was reduced in all subsequent studies to 1.63 gm. per day. The averaged results, especially those in Series IV, may appear to indicate that lysine is ketolytic (or antiketogenic), but this is due to the very high acetone body output of a single rat on a single day in each of the control series. Statistical evaluation indicates that the differences between the experimental and the control groups are not significant in either series. The greater total nitrogen excretion per day by the experimental animals in these than in the series fed lysine alone would suggest that more lysine was absorbed and metabolized. In general the findings are in accord with those on glycogenesis.

*Glutaric Acid As Ketogenic or Ketolytic (or Antiketogenic) Agent*—Ringer concluded that glutaric acid did not alter appreciably the excretion either of sugar or of the acetone bodies in the phlorhizinized dog (1912) and suggested (Ringer, Frankel, and Jonas, 1913) that glutaric acid may be a metabolite of lysine.

Preliminary tests indicated that rats would survive the feeding of only small amounts of glutaric acid each day; the 0.33 gm. dosage was apparently small enough to be non-toxic. In the initial test for ketogenesis (Series V, Table II) five rats (Group A) were fed

the acid and five (Group B) served as controls. After an intervening period of 9 days on stock diet, during which all animals approximately regained their original weights, these regimens were reversed (Series VI). Comparisons between the groups during their periods on glutaric acid feeding and their periods when fed only water, as well as comparisons between the experimental and control animals in each period, indicate a somewhat greater output of total acetone bodies after glutaric acid feeding. Excretion of glutaric acid could hardly have influenced the results; 0.6 gm. samples analyzed by the Van Slyke procedure produced precipitates equivalent to less than 0.002 gm. per sq.m. per day for rats of the size employed. Statistical treatment suggests that the differences between the output in the control and experimental animals of Series V may be significant; the absolute differences, however, seem so small as to be of little practical consequence.

The ketolytic (or antiketogenic) effect of glutaric acid was tested similarly in rats (Groups C and D) fed an equivalent amount of sodium butyrate. In both Series VII and VIII (Table II) the glutaric acid feeding lowered the average total acetone body output and raised the nitrogen excretion slightly. In Series VII the acetone body output was higher, the total nitrogen excretion lower than in Series VIII. Statistical evaluation of the results indicates that the reduction in output of acetone bodies induced by feeding glutaric acid may possibly be significant. Again, however, the differences are not striking or of the order observed in feeding substances definitely ketolytic (or antiketogenic).

#### SUMMARY

*dl*-Lysine monohydrochloride fed to fasted rats in amounts allowing maximum absorption over periods of 4, 8, and 12 hours produced no greater glycogen deposition in the liver than was found in fasted controls.

The average output of total acetone bodies in two groups of rats during a 3 day period of *dl*-lysine monohydrochloride feeding was only slightly greater than in the corresponding control groups receiving no lysine. When the *dl*-lysine monohydrochloride was fed with sodium butyrate during a 4 day period, it was found to reduce the acetone body output slightly below that obtaining in the controls. Critical study of the data and evaluation by statistical

methods do not seem to justify assuming that the differences observed in either series are significant.

Glutaric acid fed in similar tests caused similar responses. Smaller individual variations in the experimental and control groups make the differences between the two appear statistically more significant, but not strikingly so. The results obtained do not oppose the possibility that glutaric acid may be an intermediate in the metabolism of lysine.

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# ABSORPTION EXPERIMENTS WITH VITAMIN A

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Results are here reported of investigations on absorption and excretion of vitamin A with human subjects. The literature seems lacking in experimental work of this nature.

It has been found that a large percentage of the vitamin A given to subjects cannot be accounted for as such in the blood, and a search has been made for changed forms or destruction products of the vitamin. Spectrographic methods have revealed a rather broad absorption band in the blood and feces of individuals who had taken massive doses of vitamin A. This band, not shown by blood and feces of individuals on a normal diet, seems to indicate a degeneration product of vitamin A. Evidence is given here from purely chemical investigations on vitamin A concentrates of a relationship of this degradation product to vitamin A.

## EXPERIMENTAL

### *Blood and Feces Analyses*

The subjects used in these experiments were all males, 20 to 30 years of age, in good health, not under treatment nor on special diets, and, by the criterion of the Pett visual test (1), normal as to vitamin A status.

The first experiment, carried out on three individuals, involved the feeding of a relatively small dose of vitamin A.

Each subject was given a 20 grain charcoal pill. After a short interval, a blood sample was taken and 10,000 i.u. of vitamin A administered orally. The vitamin A source used was a carotene-

\* Assisted by a grant to Professor G. Hunter from the Associate Committee on Medical Research of the National Research Council, Canada.

free oil (50,000 I.U. per gm.). Further blood samples were taken after 1, 3, 5, and 8 or 24 hours. These were all analyzed for vitamin A by the method of Pett and LePage (2).

The feces were collected, and those containing the charcoal were analyzed for vitamin A and carotene. The feces analysis was carried out according to the method of von Wendt (3), with some slight modifications. Urine collected showed no vitamin A.

In the method, as used, the day's stools were mixed with anhydrous sodium sulfate and extracted with 95 per cent ethanol, first 300 ml., then three successive 100 ml. portions. For each extraction the alcohol was mixed with the sample intermittently for half an hour, then filtered off rapidly with suction. The combined extracts were mixed with 200 ml. of 60 per cent alcoholic KOH and refluxed on a boiling water bath for 10 minutes. The saponified extract was chilled, diluted to 50 per cent alcohol with distilled water, and extracted with 200 ml., then with four successive 100 ml. portions of petroleum ether.<sup>1</sup> The combined extracts were washed five times with distilled water in 100 ml. portions. The resulting extract was dried with anhydrous sodium sulfate and filtered into a graduated cylinder. Aliquots were taken for analysis.

For carotene estimation the yellow color was matched against 10 mm. of 0.02 per cent  $K_2Cr_2O_7$  solution in a colorimeter. The yellow was calculated as  $\beta$ -carotene. For the three subjects, carotene values as  $\beta$ -carotene were found to vary from 300 to 700  $\gamma$  per day's feces.

For vitamin A analysis of the feces, an aliquot of the petroleum ether extract was taken, the solvent evaporated in a stream of nitrogen, and the residue taken up in chloroform. This was mixed with chloroformic antimony trichloride in a colorimeter as described by Pett and LePage (2) and vitamin A calculated from the blue color developed. The amount of blue developed here in each case accounted only for the  $\beta$ -carotene in the feces, as the latter also gives a blue color. We thus conclude that, following the ingestion of 10,000 I.U. of vitamin A, there was no free vitamin A excreted in the feces.

<sup>1</sup> The petroleum ether used in these experiments was treated so as to be free from peroxides, aldehydes, unsaturated compounds, and aromatics, so that impurities might not complicate the spectrographic observations.

The results of the blood analysis are shown in Table I.

From the data for plasma vitamin A shown in Table I, using the assumption that the blood constitutes one-twelfth of the body weight, and that the plasma is 55 per cent of the total blood volume, we calculated the percentage of the dose given which was circulating in the blood. These percentages are also shown in Table I.

1 week later, the same three subjects, and two additional ones, were given a 20 grain charcoal pill each, blood samples were taken,

TABLE I

*Plasma Vitamin A after Feeding 10,000 International Units of Vitamin A*

Subject	Time after vitamin A given	Plasma vitamin A	
		i. u. per 100 ml.	per cent of dose
R. C.	hrs.		
	0	54	
	1	73.5	
	3	75	6.2
	5	73.5	
A. L.	24	58	
	0	47.5	
	1	47.5	
	3	61	3.9
	5	68	5.9
L. B. P.	8.2	55	
	0	70	
	1	66	
	3	86	4.7
	5	83	
	8.2	65	

and 300,000 I.U. of vitamin A were administered orally. Further blood samples were taken at 2, 4, 6, 8, and 24 hours. These were all analyzed for vitamin A. The results of this analysis are given in Table II.

The feces sample containing the charcoal and those for 2 days after were analyzed for vitamin A and carotene in each case for the three subjects. The feces analysis is given in Table III.

*Results*—As may be observed from Table II, the greatest percentage of the dose given, accounted for in the blood as vitamin A, is 3.19, in the case of L. B. P. In this same subject, the amount re-



covered in the feces is 0.46 per cent of the dose given. Thus 96.35 per cent must be considered as withdrawn by the tissues, not yet absorbed, or disposed of in other ways. While much of the vitamin

TABLE II

*Plasma Vitamin A after Feeding 300,000 International Units of Vitamin A*

Subject	Time after intake of vitamin A	Plasma vitamin A	
		<i>i. u. per 100 ml.</i>	<i>per cent of dose</i>
R. C.	<i>hrs.</i> 0	52	
	2	60	
	4	226	1.72
	6.2	96	0.43
	8	74	
	24	48	
A. L.	0	50	
	2	54.5	
	4	210	1.53
	6	83	0.31
	8	64	
	24	53	
L. B. P.	0	71	
	2	93	
	4	398	3.19
	6	382	3.04
	8	212	
	24	78	
W. D.	0	73	
	2	71.5	
	4	265	1.92
	6.1	203	1.30
	7.6	147	
	24	72	
P. B.	0	63	
	2	59	
	4	262	1.99
	6	145	0.82
	8	110	
	24	78	

A may be rapidly withdrawn by tissues, it does not seem probable that this large proportion of such a large dose could be stored in so short a time. In view of this, and the findings of Baumann, Riising, and Steenbock (4), that only 10 to 20 per cent of ad-

ministered vitamin A could be accounted for as vitamin A by analysis of rat tissues, it was decided to pursue the investigation further. The problem appeared to be one of searching for destruction products of vitamin A in the blood and feces. We began the search for such products by spectrographic examination of purified extracts of the blood and feces after administration of vitamin A.

### *Spectrographic Investigation*

*Apparatus*—A Bellingham and Stanley (England) quartz spectrograph fitted with a rotating sector mounted directly on the instrument was used. The light source is a spark between tungsten steel electrodes. Two 1 cm. cells, fitted with quartz ends, permit comparison of solution and solvent, in respect to transmission of

TABLE III  
*Vitamin A in Feces after Intake of 300,000 International Units (As  
International Units per Day)*

Subject .....	R. C.	A. L.	L. B. P.
1st day.....	1670	126	667
2nd " .....	525	1427	670
3rd " .....	0	389	65
Total.....	2195	1942	1402

the light. An adjustable sector opening behind the solvent cell compensates for the increased exposure times, and permits matching of the adjacent strip photographs for density. The actual photograph taken is about 5 inches long, covering wave-lengths from 4651 to 2210 Å. The instrument has been carefully calibrated by several standard methods. The plates are developed under standard conditions, and the curves plotted by visual comparison in special apparatus.

*Procedure*—Three subjects were again given doses of 300,000 i.u. of vitamin A. Control blood and feces samples were collected just previous to this.

A blood sample was taken from each of the three subjects 4 hours after administration of the vitamin A. The feces of each for the next 3 days were collected and pooled in each case for analysis.

Both blood and feces were analyzed for vitamin A. In addition, aliquots were extracted and prepared for spectrographic examination.

*Blood Plasma*—The aliquot of the blood plasma sample was saponified, extracted, washed, and evaporated to dryness in a stream of nitrogen at 30–40°, as in the plasma vitamin A analysis. It was then taken up in 15 ml. of absolute ethanol plus 1.5 ml. of 60 per cent alcoholic KOH. This solution was refluxed on a boiling water bath for 10 minutes, chilled, and extracted with 25, 20, and 15 ml. portions of petroleum ether. The combined extracts were washed twice with successive 20 ml. portions of distilled water, once with 10 ml. of dilute alcoholic KOH, and then with three successive 20 ml. portions of distilled water. The extract was dried with anhydrous sodium sulfate, filtered, and evaporated in a stream of nitrogen. The residue could then be taken up in the required amount of cyclohexane-ethanol mixture<sup>2</sup> for spectrographic examination.

Absorption curves for the blood extracts are shown in Fig. 1.

*Feces*—An aliquot of the feces extract in each case was evaporated to dryness in a stream of nitrogen at room temperature and the residue taken up in 10 ml. of absolute alcohol plus 1 ml. of 60 per cent alcoholic KOH. The solution was refluxed on a boiling water bath for 10 minutes, chilled, extracted with three successive portions of petroleum ether, and otherwise prepared as the blood samples were.

Absorption curves for feces extracts are shown in Fig. 2.

The same treatment was used on control blood and feces samples. The analysis data are given for these extracts in Table IV.

The absorption curves of these extracts, obtained by spectrographic examination, are shown in Figs. 1 and 2.

The concentration of the blood extracts was such that 1 ml. of cyclohexane-ethanol solution = 1 ml. of blood plasma, with the exception of the control sample of subject W. D. in which the con-

<sup>2</sup> This cyclohexane-ethanol mixture, 20 per cent cyclohexane and 80 per cent absolute ethanol, was used as solvent in spectrographic observations other than assays, because it was a better fat solvent, absolute ethanol being a poor fat solvent and not able to take up some residues rapidly. This solvent shifts the characteristic vitamin A absorption band of 328 m $\mu$  toward 310 m $\mu$ . Cholesterol absorption adds to this apparent shift.

centration was doubled. In examination of feces extracts the concentrations were such that 1 ml. of cyclohexane-ethanol solution = 1 ml. of petroleum ether extract except in the cases of subjects W. D. and L. B. P. after the vitamin dose. In the latter two cases the concentration was doubled. The volumes of the petro-

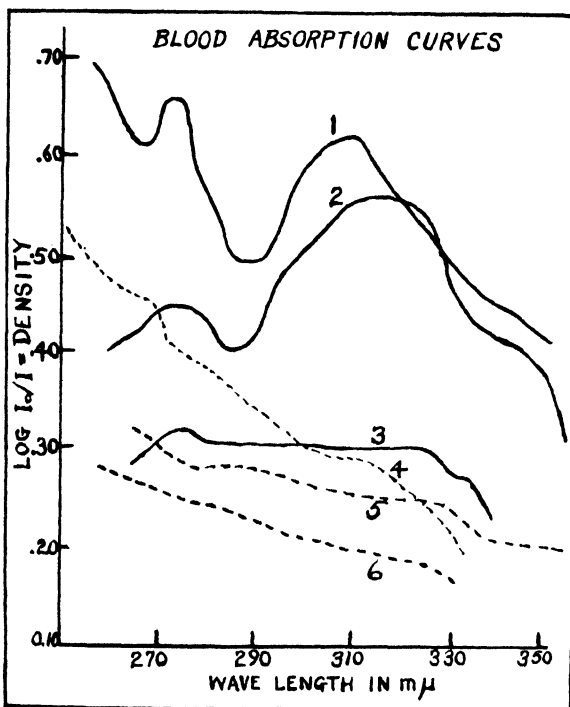


FIG. 1. Spectrographic absorption curves of blood before and after ingestion of vitamin A. Curve 1 (L. B. P.), Curve 2 (W. D.), Curve 3 (A. L.), blood after vitamin dose; Curve 4 (L. B. P.), Curve 5 (W. D.), Curve 6 (A. L.), control blood.

leum ether extracts of feces were fairly constant, being 500 ml. for single specimens, 1500 ml. for pooled 3 day specimens.

In the absorption curves, a peak is shown at 275  $m\mu$ . This is not present in control blood or control feces extracts, nor in the vitamin A concentrate used.

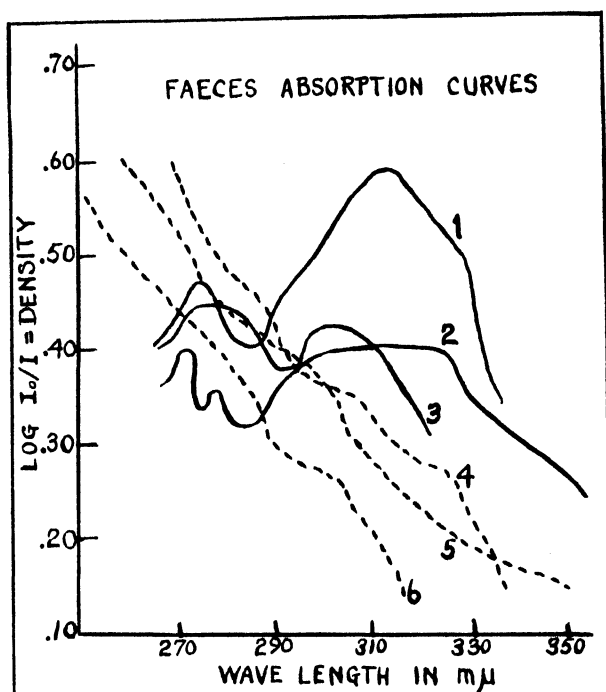


FIG. 2. Spectrographic absorption curves of feces before and after ingestion of vitamin A. Curve 1 (W. D.), Curve 2 (L. B. P.), Curve 3 (A. L.), feces after vitamin dose; Curve 4 (L. B. P.), Curve 5 (W. D.), Curve 6 (A. L.), control feces.

TABLE IV  
*Analysis Data of Extracts Used for Spectrographic Examination*

Subject	Plasma vitamin A, initial	Plasma vitamin A 4 hrs. after dose	Feces vitamin A for 3 day period
	i.u. per 100 ml.	i.u. per 100 ml.	i.u.
L. B. P.	70	876	9,820
A. L.	50	119	1,694
W. D.	60	359	13,600

*Results*—Several facts are apparent from these balance experiments. The blood analysis data show that normal individuals reach a maximum blood vitamin A level 4 to 5 hours after oral

intake of the vitamin, as was also found by de Haas and Meulemans (5) and Chesney and McCoord (6). In none of the cases observed did more than 8 per cent of the vitamin appear in the blood stream at its maximum level. More usually the proportion in the blood was much less than this. In no case did excretion of vitamin A in the feces exceed 4 per cent of that administered. The excretion was more usually of the order of 0.4 per cent.

About the same percentage of the vitamin A dose given appears in the blood whether 10,000 or 300,000 I.U. be given, but naturally the actual amount is higher with the large dose.

No vitamin A was found in the feces after doses of 10,000 I.U., but some was found after 300,000 I.U. If the amount found with the large dose is calculated for the small one, it is only 50 to 100 I.U. This amount could be missed in our procedure by calculating that all the yellow color of the extract is due to carotene. That there is an error in this assumption is indicated by the investigations of Moore (7) on bovine feces. He found 30 per cent of the color due to yellow pigments of no vitamin A activity. This error would not, however, be very significant in respect to the deductions for carotene in blood, or that for carotene in feces after large doses of vitamin A.

Blood and feces of normal individuals after large doses of vitamin A have been shown to contain a substance giving an absorption band in the ultraviolet at about 275  $m\mu$ . Since this band was not found in control feces or blood, nor in the vitamin A concentrate, it would seem that it must be attributed to some chemical entity produced metabolically from vitamin A. Further chemical evidence will be presented that such is the case and that the product is partially oxidized vitamin A.

#### *Chemical Treatment of Vitamin A Concentrates*

It was assumed that the substance showing absorption at 275  $m\mu$ , found in blood and feces after administration of large doses of vitamin A, was either an oxidation or a reduction product of the vitamin. Experiments were planned to test this hypothesis on the material used for the feeding experiments (50,000 I.U. per gm.). Eventually two other concentrates were also used. These were a ling cod liver oil (196,000 I.U. per gm.) and a shark liver oil (112,700 I.U. per gm.).

In each case a 50 mg. sample of the oil was weighed into a 50 ml. Erlenmeyer flask; 20 ml. of 95 per cent ethanol and 2 ml. of 60 per cent alcoholic KOH were added, and the solution was refluxed on a boiling water bath for 10 minutes. Then it was chilled, diluted with distilled water to 50 per cent alcohol, and extracted with 25, 20, and 20 ml. of petroleum ether. The combined extracts received washes in the extraction funnel with two successive 20 ml. portions of distilled water, with 10 ml. of 6 per cent alcoholic KOH, and finally with three successive 20 ml. portions of distilled water. The extract was dried with anhydrous sodium sulfate, filtered, and washed into a 100 ml. Erlenmeyer flask. The solvent was evaporated in a stream of nitrogen at 30–40° and the residue taken up in 20 ml. of absolute ethanol.

The above purification, when carried out on a 50 gm. sample of the shark liver oil, yielded about 6 gm. of a yellow-orange oil, having, at  $\lambda$  328 m $\mu$ ,  $E_{1\text{ cm.}}^{1\%}$  = 543, potency 870,000 I.U. of vitamin A per gm.

This solution of purified residue in 20 ml. of absolute ethanol was convenient for oxidation or reduction treatments and the reaction could be carried out in the same vessel.

Reduction was tried by addition of sodium amalgam to this alcohol solution and subsequent extraction of the products. Spectrographic examination showed that destruction of considerable vitamin A had occurred, but the only absorption band was that at 328 m $\mu$ , characteristic of vitamin A.

Oxidation was tried by addition of hydrogen peroxide to the purified samples. This reagent assayed 28.35 per cent H<sub>2</sub>O<sub>2</sub>. The procedure for this oxidation was the same for all three of the oils used, varying with the oil only in respect to the time of exposure to the oxidant. One was exposed 20 minutes; another showed the desired effects after a 35 minute exposure.

In the preliminary work with blood and feces extracts after vitamin A administration, and in these purely chemical experiments, one component at least of those absorbing in the ultra-violet proved somewhat unstable. Hence precautions were found necessary in this procedure.

To the solution of purified concentrate in 20 ml. of absolute ethanol, 5 ml. of the hydrogen peroxide were added.

The mixture was maintained at 70° in a water bath, and nitrogen

was bubbled through it. At the conclusion of the required time, the extract was diluted with an equal volume of distilled water and quickly chilled to  $-22^{\circ}$  in a freezing mixture. The solution was then extracted with 25, 20, and 20 ml. of petroleum ether. The

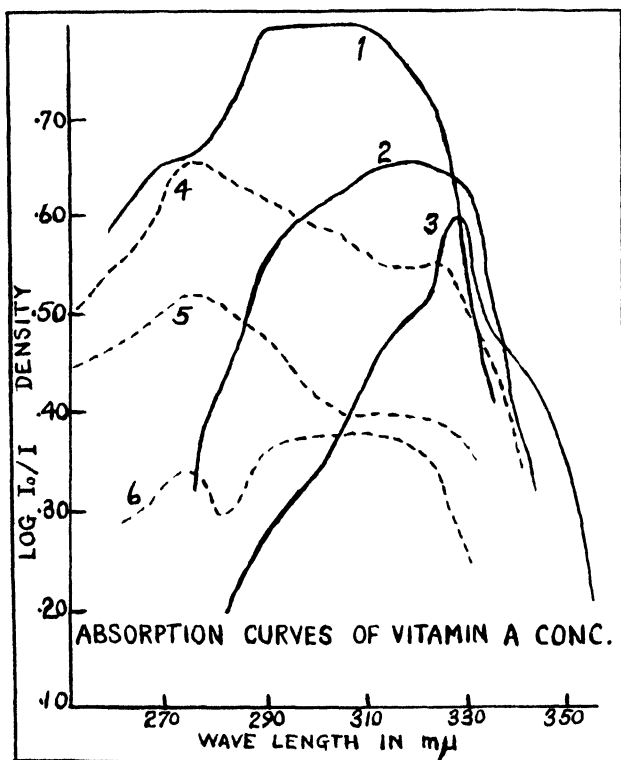


FIG. 3. Spectrographic absorption curves of vitamin A concentrates (in mg. per ml.), before and after oxidation. Curve 1, carotene-free oil, 0.2 mg.; Curve 2, shark liver oil, 0.10 mg.; Curve 3, ling cod liver oil, 0.05 mg.; Curve 4, shark liver oil after oxidation, 0.75 mg.; Curve 5, ling cod liver oil after oxidation, 0.35 mg.; Curve 6, carotene-free oil after oxidation, 0.2 mg.

combined extracts were washed five times with successive 20 ml. portions of cold distilled water, dried with anhydrous sodium sulfate, and filtered into a 100 ml. flask. In this the petroleum ether was quickly taken off under a vacuum. The residue was taken up



in cyclohexane-ethanol mixture, diluted to the required concentration, and examined spectrographically at once.

On suitable oxidation treatment with hydrogen peroxide, the three vitamin A concentrates all gave absorption curves of the same features as those obtained with blood and feces after vitamin A

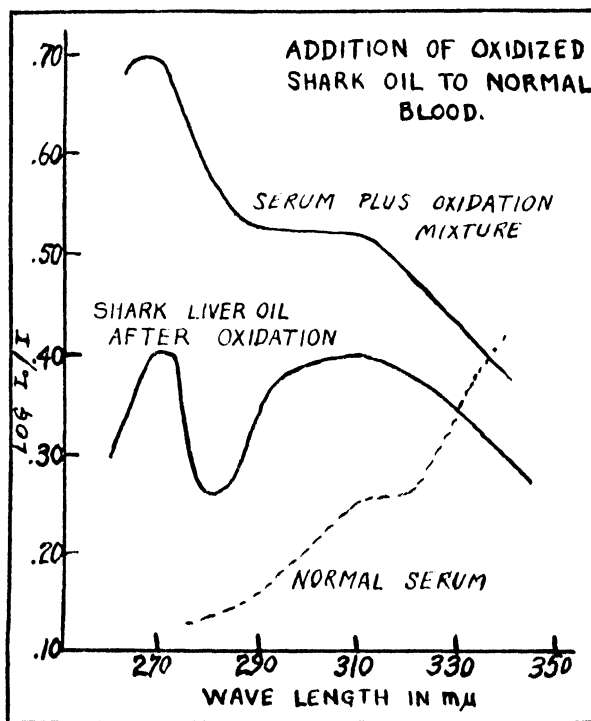


FIG. 4. Spectrographic absorption curve of an "oxidized" shark liver oil added to normal serum, and the control curves. (Taken with technical assistance from S. Sereda and K. McKerns.)

administration. That is, they all exhibited the absorption maximum due to vitamin A at 328 mμ and also a maximum in the vicinity of 275 mμ. The curves obtained for these oils before and after oxidation are shown in Fig. 3.

These curves of the oxidized material tend to lose the absorption in this region during the 1st hour or 2 after chemical treatment, sug-

gesting the lability of the substance concerned, and its further chemical change.

The possibility that steroid material, of which the chief one present is cholesterol, might be interfering was checked by obtaining absorption curves for cholesterol in concentrations similar to those involved in this work. Curves given for it accounted only for general absorptions in this region, similar to the absorption curves shown for control blood and feces samples.

Finally, suitable quantities of shark oil oxidation mixture were added to normal blood serum, extracted as above, and the absorption spectrum determined. Fig. 4 shows the curves. It is clear that such an oxidation product can be determined in blood.

*Results*—Since oxidation of a purified vitamin A concentrate produces a substance with an absorption band very similar to that of the substance in blood and feces after a vitamin A dose, it would seem that the substance in question is an oxidation product of vitamin A.

In work on vitamin A assays, Morton and Heilbron (8) report that one of the first decomposition products on oxidation of vitamin A exhibits an absorption band at 275 to 285  $m\mu$ . Edisbury and Morton (9) found a material in a vitamin A concentrate which was insoluble in 83 per cent methanol and showed a bioassay too high to be residual vitamin A. This fraction gave an absorption band at 285 to 290  $m\mu$ . Castle *et al.* (10) report obtaining from vitamin A concentrates a substance absorbing at 270 to 280  $m\mu$  which they state to be an oxidation product of vitamin A. Karrer *et al.* (11) separated a highly concentrated vitamin A into two fractions, one absorbing at 328  $m\mu$ , the other at 270  $m\mu$ . Edisbury *et al.* (12) report that ozone attacks vitamin A with production of, first, a compound having an absorption maximum at 290  $m\mu$ , then one with a maximum at 272  $m\mu$ . On the basis of similar structures, the latter authors suggest that ozone must attack a double bond in the side chain, then another, both selectively. These references all seem to confirm the suggestion that the chemical identity of the product found absorbing at 275  $m\mu$  is an oxidation product of vitamin A.

Under the conditions where it was found in both blood and feces, at least two explanations are possible, and both should be

considered. Either the oxidation product is produced in the blood after absorption, in which case some must be excreted again, or it is produced in the gastrointestinal tract and part of it absorbed. A study of individuals with various abnormalities, such as liver dysfunction and faulty fat absorption, might throw some light on this point.

Criticism could be offered that perhaps the source of this material in blood and feces extracts was oxidation during the purification procedures. However, this possibility is small when it is considered that the oils which were examined after the same procedure of saponification, etc., did not show any such characteristic absorption. Also, the calculation of vitamin A in blood and feces from spectrographic plates agreed with the assays obtained by colorimetric measurements previous to this purification.

#### SUMMARY

1. Quantitative experiments on the absorption of orally administered vitamin A by humans indicate that 88 to 96 per cent of the vitamin ingested cannot be accounted for in blood and feces as vitamin A.

2. A substance was found in blood and feces after ingestion of large doses of vitamin A which seems to be a closely related oxidation product.

3. Strong evidence that this related substance is an oxidation product of vitamin A has been obtained by purely chemical investigation.

4. The nature of the oxidizing system and the importance of the fate of the oxidized product remain to be determined.

5. Incidental to the work, blood levels of vitamin A during absorption from the intestinal tract have been determined for several persons.

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# ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

## III. THE PROTEOLYTIC ENZYMES OF BEEF SPLEEN, BEEF KIDNEY, AND SWINE KIDNEY. CLASSIFICATION OF THE CATHEPSINS

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Aqueous extracts of beef spleen have been shown to contain at least three proteolytic enzymes of widely different specificity (1). Beef spleen Cathepsin I hydrolyzes carbobenzoxy-*l*-glutamyl-*l*-tyrosine and does not require the addition of an activator such as cysteine. Beef spleen Cathepsin II hydrolyzes benzoyl-*l*-arginineamide when an activator such as cysteine is added. Beef spleen Cathepsin III hydrolyzes *l*-leucineamide in the presence of activators such as cysteine or ascorbic acid. In the present communication, the identification of a fourth proteolytic component (Cathepsin IV) of beef spleen cathepsin is reported. Furthermore, counterparts of these four enzymes have also been found in beef kidney and swine kidney.

### *Identification of a New Proteolytic Enzyme in Beef Spleen Cathepsin*

An indication for the presence of a fourth proteolytic component in beef spleen cathepsin was obtained by a comparative study of the action of a cysteine-activated spleen extract upon benzoyl-*l*-arginineamide, benzoyl-*l*-lysineamide, carbobenzoxyglycyl-*l*-phenylalanine, and carbobenzoxyglycyl-*l*-tyrosine. It was previously reported (2) that Cathepsin II is unstable at pH values more acid than 4. It will be noted from the data in Table I that the activity of beef spleen cathepsin toward carbobenzoxyglycyl-phenylalanine is more resistant to acidity than is the activity of

Cathepsin II. It must be concluded that carbobenzoxyglycyl-*l*-phenylalanine is hydrolyzed by a cysteine-activatable enzyme other than Cathepsin II. This component is the Cathepsin IV mentioned above. The data in Table I indicate also that at pH 3.5 and 3.9 the activities of spleen cathepsin toward benzoyl-arginineamide and benzoyllysineamide decreased in a parallel manner. This indicates that benzoyllysineamide is split by the same enzyme as is benzoylarginineamide; *i.e.*, Cathepsin II.

TABLE I

*Effect of Increased Acidity on Activity of Beef Spleen Cathepsin*

Samples of a solution of beef spleen cathepsin (1.40 mg. of protein N per cc. of enzyme solution) were adjusted to pH 3.9 or 3.5 with *N* acetic acid and kept at these pH values for 1 hour at 40°. The pH of the enzyme solutions was then brought to 5.0 with *N* NaOH. In a control experiment a mixture of *N* acetic acid and *N* NaOH was made up in the proportions applied above and then added to the enzyme. The control enzyme solution was also kept at 40° for 1 hour. The three above enzyme solutions were tested for enzymatic activity in the presence of cysteine (0.01 mm per cc. of test solution). The pH of the test solution was 4.8; the temperature was 40°.

Substrate	$C^* \times 10^3$		
	Control	Treated enzyme	
		pH 3.9	pH 3.5
Benzoyl- <i>l</i> -arginineamide (4) .....	8.3	5.2	2.8
Benzoyl- <i>l</i> -lysineamide (5).....	4.0	2.4	1.3
Carbobenzoxyglycyl- <i>l</i> -phenylalanine (6).....	4.2	4.1	3.5

$$* C = \frac{K \text{ (first order)}}{\text{mg. protein N per cc. test solution}} \quad (3).$$

A sample of beef spleen cathepsin was dialyzed against distilled water, and its activity upon the previously mentioned substrates was tested before and after dialysis. The data in Table II show that on dialysis the proteolytic coefficient toward benzoylarginineamide rises slightly,<sup>1</sup> while the proteolytic coefficients toward carbobenzoxyglycylphenylalanine and carbobenzoxyglycyltyrosine drop markedly. This decrease is of the same magnitude for both substrates. It may therefore be concluded that the hydrolysis of carbobenzoxyglycylphenylalanine and carbobenzoxyglycyltyrosine is due to the same enzymatic component of spleen cathepsin; *i.e.*, Cathepsin IV.

<sup>1</sup> This increase is due to the loss of protein other than Cathepsin II.

It had previously been found (1) that carbobenzoxy-*l*-glutamyl-*l*-phenylalanine is hydrolyzed by two different enzymatic components of beef spleen cathepsin, one of which (Cathepsin I) is effective in the absence of an added activator, while the other is activated by cysteine. It has now been found that the activity of this second, cysteine-activatable enzyme was not diminished when the beef spleen extract was kept at pH 3.9 and 40° for 1 hour, but that this enzyme is lost when the spleen extract is dialyzed against distilled water for 48 hours. This behavior is similar to that previously found for Cathepsin IV, and it may be assumed that the cysteine-activatable beef spleen component that acts on carbobenzoxyglutamylphenylalanine is identical with the component

TABLE II

*Loss of Cathepsin IV Activity on Dialysis of Beef Spleen Cathepsin*

A solution of beef spleen was dialyzed against 1 per cent sodium chloride. The resulting Solution A was then dialyzed against distilled water for 48 hours at 4° to give an enzyme Solution B. Solutions A and B were tested for proteolytic activity with cysteine as activator (0.01 mm per cc. of test solution). Temperature, 40°; pH 4.8 to 5.1.

Substrate	$C \times 10^3$		Cathepsin component
	Enzyme Solution A	Enzyme Solution B	
Benzoyl- <i>l</i> -arginineamide .....	8.0	9.1	II
Carbobenzoxyglycyl- <i>l</i> -phenylalanine .....	3.7	1.4	IV
Carbobenzoxyglycyl- <i>l</i> -tyrosine (7) .....	2.3	0.9	"

that hydrolyzes carbobenzoxyglycylphenylalanine; namely, Cathepsin IV. This tentative conclusion cannot, at present, be subjected to a decisive test by comparing, under various experimental conditions, the reaction rates of the cysteine-activated catheptic hydrolyses of carbobenzoxyglycylphenylalanine and carbobenzoxyglutamylphenylalanine. Since the latter substrate is hydrolyzed simultaneously by two spleen components, rate constants for its hydrolysis cannot be calculated.

*Cathepsins of Beef Kidney and Swine Kidney*

*Cathepsin I*—Similarly to beef spleen, extracts of beef kidney and swine kidney contain enzymes (beef kidney Cathepsin I and swine kidney Cathepsin I) that hydrolyze carbobenzoxy-*l*-glutamyl-*l*-tyrosine in the absence of added activators. These



enzymes are rapidly inactivated at 50°. This thermolability was utilized to show that Cathepsin I in beef kidney and swine kidney hydrolyzes not only carbobenzoxyglutamyltyrosine but also the compound carbobenzoxy-*l*-glutamyl-*l*-phenylalanine. It will be noted in Table III that after the enzyme solutions were heated at 50° for 15 minutes the activity toward the two substrates decreased to the same degree.

*Cathepsin II*—The substrate for cysteine-activated beef spleen Cathepsin II, benzoyl-*l*-arginineamide, is also hydrolyzed by beef kidney and swine kidney extracts after cysteine has been added as the activator. The enzymes responsible for these hydrolyses are

TABLE III  
*Heat Inactivation of Cathepsin I*

Solution A of beef kidney cathepsin (1.55 mg. of protein N per cc. of enzyme solution) was heated at 50° for 15 minutes and then was chilled in ice water to give a beef kidney cathepsin solution (B). A Solution A of swine kidney cathepsin (1.10 mg. of protein N per cc. of enzyme solution) was also treated as above to yield a swine kidney cathepsin solution (B). 0.5 cc. of the enzyme Solutions A and B was employed for the hydrolytic experiments. No cysteine was added. Temperature, 25°; pH 5.3 to 5.5.

Enzyme solution	Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine (8)		Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	
	$K \times 10^4$	$\frac{K(B)}{K(A)}$	$K \times 10^4$	$\frac{K(B)}{K(A)}$
Beef kidney, A . . . . .	6.2		2.6	
“ “ B . . . . .	3.0	0.48	1.2	0.46
Swine “ A . . . . .	7.0		3.5	
“ “ B . . . . .	5.3	0.76	2.6	0.74

designated beef kidney Cathepsin II and swine kidney Cathepsin II. The first order velocity constants for the hydrolysis effected by these enzymes are proportional to the enzyme concentration within the limits employed (Table IV). The value of the proteolytic coefficient for benzoylarginineamide ( $C_{BAA}$ ) for beef spleen extract was previously found to be about 0.008, corresponding to 4 enzyme units per mg. of protein nitrogen, when an enzyme unit is defined according to a previous paper (3). On the same basis, the beef kidney extract used in the experiments reported in Table IV contained 3.6 units per mg. of protein nitrogen, while the swine kidney extract contained 12 units per mg. of protein nitrogen.

Beef kidney Cathepsin II and swine kidney Cathepsin II were also found to hydrolyze benzoyl-*l*-lysineamide. The experimental data will be presented in a succeeding section of this paper.

*Cathepsin III*—Both beef kidney and swine kidney extracts contain an enzyme (Cathepsin III) that hydrolyzes *l*-leucineamide and *l*-leucylglycine at pH 5 when ascorbic acid or cysteine has been

TABLE IV

*Hydrolysis of Benzoyl-l-arginineamide by Cathepsin II of Beef and Swine Kidney*

0.01 mM of cysteine per cc. of test solution. Temperature, 40°; pH 4.7.

Source of enzyme	Protein N per cc. test solution	$K \times 10^4$	$C \times 10^4$
	mg.		
Beef kidney	0.13	9	6.9
	0.20	14	7.0
	0.33	24	7.3
Swine kidney	0.05	12	24
	0.10	26	26
	0.21	51	24

TABLE V

*Comparison of Cathepsin III Activity in Beef Spleen, Beef Kidney, and Swine Kidney*

Temperature, 40°; pH 5.0 to 5.2.

Substrate	Activator, 0.01 mM per cc. test solution	Beef spleen cathepsin*		Beef kidney cathepsin†		Swine kidney cathepsin†	
		$K \times 10^4$	$C \times 10^4$	$K \times 10^4$	$C \times 10^4$	$K \times 10^4$	$C \times 10^4$
<i>l</i> -Leucineamide	Cysteine	33	9.2	3.0	1.0	11	3.7
	Ascorbic acid	7	1.9	0.6	0.2	2.2	0.7
<i>l</i> -Leucylglycine	Cysteine	16	4.4	1.2	0.4	5	1.7

\* 0.36 mg. of protein N per cc. of test solution.

† 0.30 mg. of protein N per cc. of test solution.

added as activator (Table V). It will be noted that the Cathepsin III activity per mg. of protein nitrogen is much smaller in beef and swine kidney than in beef spleen.

*Cathepsin IV*—Extracts of beef kidney and swine kidney have been found to contain cysteine-activatable enzymes (beef kidney Cathepsin IV and swine kidney Cathepsin IV) that hydrolyze

carbobenzoxyglycylphenylalanine. Swine kidney extracts show a particularly high Cathepsin IV activity. It may be calculated from the data in Table VI that beef spleen extracts contain 1.5 Cathepsin IV units per mg. of protein nitrogen, beef kidney 3 Cathepsin IV units, and swine kidney 16.5 Cathepsin IV units.

TABLE VI

*Hydrolysis of Carbobenzoxyglycyl-L-phenylalanine by Beef Spleen, Beef Kidney, and Swine Kidney*

Cysteine, 0.01 mm per cc. of test solution. Temperature, 40°; pH 4.9.

Source of enzyme	Protein N per cc. test solution	$K \times 10^4$	$C \times 10^4$
	mg.		
Beef spleen	0.14	4	2.9
	0.28	8	2.9
	0.56	15	2.7
" kidney	0.21	12	5.7
	0.28	16	5.7
	0.42	25	6.0
Swine "	0.05	16	32
	0.10	34	34
	0.21	71	34

TABLE VII

*Effect of Cysteine on Hydrolysis of Carbobenzoxy-L-glutamyl-L-tyrosine*

Temperature, 25°; pH 5.3 to 5.5.

Source of enzyme	Protein N per cc. test solution	Hydrolysis in 2 hrs.	
		No cysteine	Cysteine*
	mg.	per cent	per cent
Beef spleen.....	0.29	22	24
" kidney.....	0.31	15	37
Swine " .....	0.18	10	36

\* 0.01 mm per cc. of test solution.

In an earlier section of this paper evidence was presented to indicate that Cathepsin IV, when activated, hydrolyzes the typical substrates of Cathepsin I (carbobenzoxy-L-glutamyl-L-phenylalanine and carbobenzoxy-L-glutamyl-L-tyrosine). This explains why tissues that are relatively rich in Cathepsin IV show a marked

increase in the hydrolysis of carbobenzoxyglutamyltyrosine on addition of cysteine (Table VII). Beef spleen, which is relatively poor in Cathepsin IV, shows no appreciable increase in the hydrolysis of carbobenzoxyglutamyltyrosine when cysteine is added.

*Classification of Cathepsins in Revised System of Proteolytic Enzymes*

In the past, proteolytic enzymes were classified as dipeptidases, polypeptidases, and proteinases (9). Consequently, the catheptic enzymes, as classified according to this scheme, included one dipeptidase, one aminopolypeptidase, one carboxypolypeptidase, and one proteinase (10). However, during recent years information has been gathered that requires revision of the above classification. In particular, the specificity and mechanism of the action of proteinases has been studied further (11). Moreover, it has been observed repeatedly that aminopeptidases and carboxypeptidases may also split dipeptides, and consequently the existence of a separate group of dipeptidases may be questioned (6, 12-14).

The revised classification proposed in Table VIII is based on the nature and position of the chemical groups in the peptide chain of the substrate that are required for the action of various proteolytic enzymes. However, the sensitivity of a substrate to a given proteolytic enzyme is determined not only by the groups in the peptide chain of the substrate but also by the nature of the constituent amino acids. Consequently, each of the classes in Table VIII may be subdivided further on the basis of the amino acid residues in the substrates that are essential for the action of specific enzymes.

It will be noted that pepsin and trypsin belong to the group of carbonylproteinases, while chymotrypsin is an imidoproteinase. The Cathepsins I of beef spleen, beef kidney, and swine kidney fall into the group of carbonylproteinases. This conclusion is based on the fact that glutamyltyrosine is not split by these enzymes and no substrate has been found for them that does not have a peptide linkage adjacent to the carbonyl side of the sensitive peptide bond. Cathepsin II is also a carbonylproteinase. Cathepsin III splits *l*-leucineamide besides *l*-leucylglycine and therefore must be an aminopeptidase. Cathepsin IV appears to be a carboxypeptidase, since carbobenzoxyglycylphenylalanineamide is not split appreciably by a cysteine-activated swine kidney extract that is rich

TABLE VIII  
*Revised Classification of Proteolytic Enzymes*

Class	Enzyme	Requisite groups in peptide chain
Peptidases (exopeptidases (15))		
Aminopeptidases	Intestinal aminopeptidase Cathepsin III	$\begin{array}{c} \text{R} \\   \\ \text{NH}_2 \cdot \text{CH} \cdot \text{CO} \text{---} \text{NH} \cdots \\ \Downarrow \end{array}$
Carboxypeptidases	Pancreatic carboxypeptidase Cathepsin IV	$\begin{array}{c} \text{R} \\   \\ \text{NH}_2 \cdot \text{CH} \cdot \text{COOH} + \text{NH}_2 \cdots \\ \text{---} \text{CO} \text{---} \text{NH} \cdot \text{CH} \cdot \text{COOH} \\   \\ \text{R} \\ \Downarrow \\ \text{---} \text{COOH} + \text{NH}_2 \cdot \text{CH} \cdot \text{COOH} \end{array}$
Proteinases (endopeptidases (15))		
Carboxylproteinases	(a) Pepsin Cathepsin I	$\begin{array}{c} \text{R} \\   \\ \cdots \text{CO} \text{---} \text{NH} \cdot \text{CH} \cdot \text{CO} \text{---} \text{NH} \cdots \\ \Downarrow \end{array}$
	(b) Trypsin Papain* Cathepsin II	$\begin{array}{c} \text{R} \\   \\ \cdots \text{CO} \text{---} \text{NH} \cdot \text{CH} \cdot \text{COOH} + \text{NH}_2 \cdots \end{array}$
Imidoproteinases	Chymotrypsin†	$\begin{array}{c} \text{R} \\   \\ \cdots \text{CO} \text{---} \text{NH} \cdot \text{CH} \cdot \text{CO} \text{---} \text{NH} \cdots \\ \Downarrow \\ \text{R} \\   \\ \cdots \text{COOH} + \text{NH}_2 \cdot \text{CH} \cdot \text{CO} \text{---} \text{NH} \cdots \end{array}$

\* The component of papain that hydrolyzes benzoyl-*l*-arginineamide.

† Chymotrypsin is designated an imidoproteinase because it hydrolyzes *l*-tyrosylglycineamide at the peptide linkage joining the tyrosyl and glycyl residues (unpublished experiments) and also hydrolyzes carbobenzoxy-tyrosylglycineamide (7).

in Cathepsin IV and highly active toward carbobenzoxyglycyl-phenylalanine.

Additional support for this classification has been obtained by comparing the action of several enzymes upon two test substrates in a quantitative manner. Thus, the Cathepsins II of beef spleen, beef kidney, and swine kidney are compared with respect to their action on benzoylarginineamide and benzoyllysineamide (Table IX). The proteolytic quotient  $C_{BAA}/C_{BLA}$  was found to be essentially the same (2.2 to 2.5). New determinations of the reaction velocity constants for the hydrolysis of the above substrates by

TABLE IX

*Hydrolysis of Benzoyl-L-arginineamide and Benzoyl-L-lysineamide by Several Proteolytic Enzymes*

In all cases, except that of trypsin, cysteine was present in a concentration of 0.01 mm per cc. of test solution and the temperature was 40°. In the experiments with trypsin, the temperature was 25°.

Enzyme	pH	$C \times 10^3$		$\frac{C_{BAA}}{C_{BLA}}$
		Benzoylarginineamide	Benzoyllysineamide	
Beef spleen Cathepsin II.....	4.7	8.3	3.8	2.2
“ kidney “ “.....	4.7	8.7	3.7	2.3
Swine “ “ “.....	4.7	27	11	2.5
Trypsin.....	7.4	42	20	2.1
Papain.....	5.0	167	78	2.1

crystalline beef trypsin (*cf.* also (5)) gave a proteolytic quotient  $C_{BAA}/C_{BLA}$  of 2.1. Furthermore, with cysteine-papain a proteolytic quotient of  $C_{BAA}/C_{BLA}$  of 2.1<sup>2</sup> was obtained.

The similarity of the proteolytic quotients  $C_{BAA}/C_{BLA}$  for the five enzymes is the more striking since the enzymes compared differ in their pH optimum and activation behavior. In our opinion, this similarity exists because the five enzymes all act upon their

<sup>2</sup> It was found that the proteolytic activity toward each substrate dropped to 30 per cent of the original value after treatment of a papain solution at pH 2 for 17 hours at 21°. The fact that the quotient  $C_{BAA}/C_{BLA}$  was unchanged indicates that the two substrates are split by the same enzymatic component of papain.

substrates by a similar reaction mechanism; they all are carbonylproteinases.

The hydrolysis of the substrates benzoylglycyl-*l*-arginineamide and benzoylglycyl-*l*-lysineamide by crystalline trypsin has already

TABLE X

*Comparison of Cathepsins IV with Crystalline Carboxypeptidase*

In all cases, except that of carboxypeptidase, cysteine was present in a concentration of 0.01 mm per cc. of test solution and the temperature was 40°. In the experiments with carboxypeptidase, the temperature was 25°.

Enzyme	pH	$C \times 10^4$		$\frac{C_{CGP}}{C_{CGT}}$
		Carbo- benzox- glycyl- <i>l</i> - phenylala- nine	Carbo- benzox- glycyl- <i>l</i> - tyrosine	
Beef spleen Cathepsin IV . . . . .	5.0	2.5	1.5	1.7
“ kidney “ “ . . . . .	5.1	6.3	4.0	1.6
Swine “ “ “ . . . . .	5.0	34	19	1.8
Carboxypeptidase* . . . . .	7.7	6570	3620	1.8

\* This preparation was kept at 0° for over 1 year and thus had lost some activity.

TABLE XI

*Comparison of Cathepsin I Activity in Beef Spleen, Beef Kidney, and Swine Kidney*

No cysteine was added. Temperature, 25°; pH 5.3 to 5.5.

Source of enzyme	Protein N per cc. test solution	Carbobenzoxyl- <i>l</i> -glu- tamyl- <i>l</i> -phenylalanine		Carbobenzoxyl- <i>l</i> - glutamyl- <i>l</i> -tyrosine		$\frac{C_{CGP}}{C_{CGT}}$
		$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$	
	mg.					
Beef spleen . . . .	0.45	7.2	1.6	14.7	3.3	0.48
“ kidney . . . .	0.31	2.6	0.84	6.2	2.0	0.42
Swine “ . . . .	0.22	3.5	1.6	7.0	3.2	0.50

been studied (16) and the quotient  $C_{BGAA}/C_{BGLA}$  was found to be 1.8. This value is close to those given above for the quotient  $C_{BAA}/C_{BLA}$ .

In Table X the Cathepsins IV of beef spleen, beef kidney, and swine kidney as well as crystalline carboxypeptidase from beef

pancreas are compared with respect to their action on carbobenzoxyglycyl-*l*-phenylalanine and carbobenzoxyglycyl-*l*-tyrosine. It will be noted that the proteolytic quotient  $C_{CGP}/C_{CGT}$  was found to be 1.6 to 1.8. Here again enzymes are compared which differ with respect to pH optima and activation behavior but which belong to the same group of carboxypeptidases.

The proteolytic quotients  $C_{CGP}/C_{CGT}$  for the hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine and carbobenzoxy-*l*-glutamyl-*l*-tyrosine by the Cathepsins I of beef spleen, beef kidney, and swine kidney were found to be 0.42, 0.48, and 0.50, respectively (Table XI), thus indicating that the phenylalanine-containing substrate is hydrolyzed at a rate one-half of that of the tyrosine-containing substrate. It will be recalled that in the case of Cathepsin IV the phenylalanine-containing substrate was hydrolyzed at nearly twice the rate of the hydrolysis of the tyrosine-containing substrate. This difference in the quotients for Cathepsin I and Cathepsin IV indicates that these two enzymes have different mechanisms of action, and serves as added support for the classification of Cathepsin I and Cathepsin IV in separate classes (Table VIII).

The authors wish to express their thanks to Mr. Maurice Rapport for valuable assistance in this investigation.

#### EXPERIMENTAL

The beef spleen cathepsin solutions were prepared as described in a previous paper (2). The same procedure was employed for the preparation of beef kidney cathepsin and swine kidney cathepsin. Crystalline trypsin was prepared according to the directions of Kunitz and Northrop (17). Crystalline carboxypeptidase was prepared by the method of Anson (18). The papain preparation was obtained as described in a previous paper (19).

The course of enzymatic hydrolysis was followed by means of amino nitrogen determinations and the microtitration method of Grassmann and Heyde. The substrate concentration was 0.05 mM per cc. of the test solution in all cases. The pH was adjusted by means of citrate buffers (near pH 5) and by means of phosphate buffers (near pH 7).



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# STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

## IV. THE SUCCINOXIDASE SYSTEM\*

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The "homogenization" method for studying tissue oxidations (1) has been used for the study of the succinoxidase system (2, 3) because it affords a simple method for eliminating side reactions, endogenous respiration, and superimposed effects due to further breakdown of fumarate. It has not been possible heretofore to know whether an enzyme has been damaged during the course of its preparation, because the determination of the amount of the enzyme in the original intact tissue has not been made. The present study attempts to answer this question and represents a further application of the homogenization technique to the study of the succinoxidase system in the light of facts which have become available since the earlier publication. The two main points of the paper have arisen from the fact that cytochrome *c* can now be prepared in relatively pure form (4), and the fact that methylene blue has been shown to stimulate some succinoxidase systems and to inhibit others (5). The succinoxidase system as it is now known is made up of at least three components; namely, succinic dehydrogenase, cytochrome *c*, and cytochrome oxidase.

### EXPERIMENTAL

*Enzyme*—Rat liver which had been freshly homogenized and diluted with 9 volumes of ice-cold M/30 sodium phosphate, pH 7.4, was used unless otherwise indicated. This material will be referred to as a "homogenate."

\* This investigation was supported by the Jonathan Bowman Fund for Cancer Research.

*Substrates*—Eastman sodium succinate was brought to pH 7.4 before use. Merck's hydroquinone was recrystallized from water.

*Cytochrome c*—The preparation was carried out according to Keilin and Hartree (4) except that the final product was dialyzed against distilled water instead of 1 per cent sodium chloride, since it was found that chloride inhibits the succinoxidase system.

*Apparatus*—Oxygen uptake was measured in a conventional Warburg apparatus at 24°, which was the temperature at which the spectrophotometric rate measurements were carried out. Alkali cups were used except when otherwise specified, and in these cases parallel experiments proved they were unnecessary. Side arms were not used except in experiments with hydroquinone. All results are reported in terms of the  $Q_{O_2}$ ; i.e., oxygen uptake per mg. of dry tissue per hour. Readings were taken every 10 minutes and the linear rate was chosen. The rate of cytochrome *c* reduction was studied by means of a Cenco-Sheard spectrophotometer (6, 7).

#### *Cytochrome c and Dilution Effect*

At a tissue dilution of about 1:100 the endogenous respiration is abolished and added succinate is oxidized to fumarate at a rate which is proportional to the amount of tissue added. Potter and Elvehjem defined the dilution effect as "the lowering of the  $Q_{O_2}$  which occurs when tissue suspensions are diluted" (1) and observed that it occurred in the case of endogenous respiration and in the presence of added glucose or lactate but not in the case of added succinate (1, 2). Elliott and Greig (3) confirmed the latter point. Since it is generally agreed that cytochrome *c* is a part of the succinoxidase system (3) and since this compound is water-soluble, one would expect to obtain a dilution effect in the case of the succinoxidase system owing to the diffusion of cytochrome *c* away from the tissue particles. Moreover, added cytochrome would be expected to raise the  $Q_{O_2}$  of the system. The data in Fig. 1 confirm the latter prediction but not the former. It is demonstrated that cytochrome *c* raises the  $Q_{O_2}$  of the preparation,<sup>1</sup> but since the same  $Q_{O_2}$  is observed with different amounts of the homogenate no dilution effect is demonstrated by these data. Since it is not pos-

<sup>1</sup> In the presence of succinate; the endogenous respiration is not raised by the addition of cytochrome *c* to this preparation.

sible to measure the  $Q_{O_2}$  of concentrated suspensions of the homogenate owing to limitations in the rate of oxygen diffusion, the dilution effect can only be studied over a rather narrow range of tissue concentrations when homogenates are used. In order to determine whether a dilution effect had occurred when intact cells were homogenized, it therefore became necessary to compare the rate of oxygen uptake due to the succinoxidase system in intact

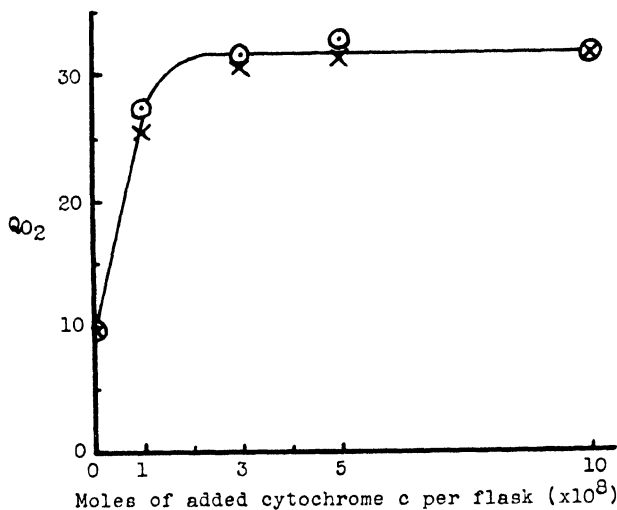


FIG. 1. The effect of cytochrome *c* on the succinoxidase system in a liver homogenate at dilutions of 1:100 and 1:150. Each flask contained 0.3 ml. of 0.25 M sodium phosphate, pH 7.4, 0.3 ml. of 0.5 M sodium succinate, pH 7.4, plus various amounts of  $10^{-4}$  M cytochrome *c* as indicated, plus 0.2 ml. (indicated by  $\times$ ) or 0.3 ml. (indicated by  $\circ$ ) of 10 per cent liver homogenate, plus water to make 3.0 ml.

cells with the same system in comparable homogenized cells. Although it is not possible to rule out endogenous respiration and side reactions in the intact cells, it is possible to get a maximum  $Q_{O_2}$  which one would not expect to exceed with disintegrated cells. Data from such an experiment are shown in Fig. 2. The minced liver was obtained by using a special mincer<sup>2</sup> adjusted to give the critical particle size needed to permit adequate inward diffusion of

<sup>2</sup> Seevers, M. H., and Shideman, F. E., *Science*, **94**, 351 (1941).

oxygen with minimum loss of cytochrome due to outward diffusion. That both conditions were well met is demonstrated by the data. A comparison of the minced tissue with the homogenized tissue in the absence of added cytochrome shows that a marked loss of activity has occurred in the case of the disintegrated cells. That this loss in activity is only a simple dilution effect is demonstrated by the fact that the addition of cytochrome *c* to the homo-

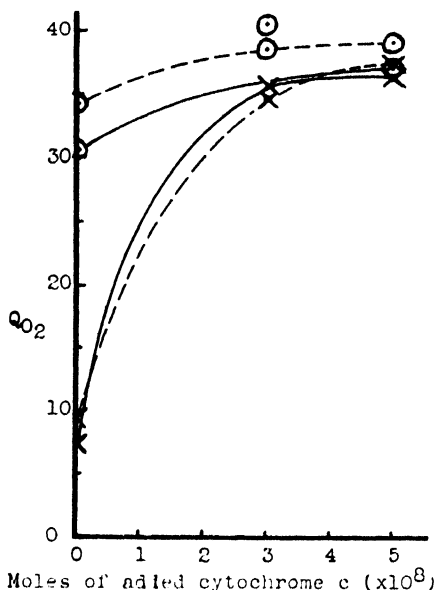


FIG. 2. Evidence for a dilution effect in the succinoxidase system due to a loss of cytochrome *c*. Minced tissue ( $\odot$ ), *versus* homogenized tissue ( $\times$ ), in air (continuous line) and in oxygen (broken line). Each flask contained phosphate, succinate, and water as in Fig. 1, plus cytochrome as indicated, plus homogenate or mince equivalent to 20 mg. of fresh liver.

genate restores the  $Q_{O_2}$  approximately to the level of the intact cells. The latter value here obtained is in fairly good agreement with the values obtained by Rosenthal (8) with liver slices.

The previous absence of a dilution effect (1-3) as well as that deduced from Fig. 1 may be explained on the assumption that a homogenate consists of particles of protoplasm which can be classified into two main categories: (*a*) particles which are so large that

cytochrome *c* cannot diffuse out and thus maintains an effective local concentration and (b) particles which are small enough so that the cytochrome *c* is lost into the medium. It should be pointed out that the homogenate contains very few intact cells or nuclei although it is increasingly apparent that the word "homogenized" is not altogether applicable, and "disintegrated" would be a better term. In addition to the two types of particles listed above it appears that there are particles with active centers on the outside which lack cytochrome *c* and active centers on the inside with cytochrome *c in situ*. According to the above explanation the succinate  $Q_{O_2}$  in the absence of added cytochrome is due to the cytochrome originally contained in the liver and is occurring mainly in the larger particles, which are not affected by dilution.

We shall now mention a number of facts, none of which proves the above hypothesis but which collectively support it. It is of course implicit in the hypothesis that all of the hydrogen transport proceeds via cytochrome *c*, and that even in the absence of added cytochrome *c* the transport is brought about by the cytochrome *c* which is contained in the system. Up to the present no investigator has been able to state the cytochrome content of such a system. However, we have recently developed a quantitative method for cytochrome *c* and on the basis of analyses made by Mr. K. P. DuBois it can be shown that the cytochrome *c* content of each flask, when none is added separately, is about 3  $\gamma$  or approximately  $2 \times 10^{-10}$  mole when 30 mg. of liver are used. From this it can be calculated that the amount of cytochrome *c* required to saturate the dispersed succinoxidase system is such that the resulting cytochrome molarity in the buffer medium approximates the cytochrome molarity in the intact liver cell. This fact is considered to be rather significant. The "turnover number" of cytochrome *c* (9) in the intact cell is about 4000 according to these data. The fact that it is considerably lower in the case of the homogenized cells is by no means a reflection of damage to the enzymes involved but is simply due to the fact that they are dispersed in a larger volume.

Given the amount of cytochrome originally present in the liver tissue, one can plot  $Q_{O_2}$  against total cytochrome instead of added cytochrome, as has been done in Figs. 1 and 2. This has been done in Fig. 3, with a greatly expanded abscissa, and it is apparent that

the  $Q_{O_2}$  does not extrapolate to zero at zero cytochrome concentration. This observation is also readily explained on the basis of variation in particle size.

The hypothesis will also explain certain paradoxical results which were obtained when the rate of cytochrome *c* reduction was measured spectrometrically. When these rates were calculated in terms of oxygen uptake, they were always lower than the observed rates as measured in the Warburg apparatus, although the rate of reduction of cytochrome *c* should not be slower than the over-all rate of oxygen uptake. Although this observation as well as the

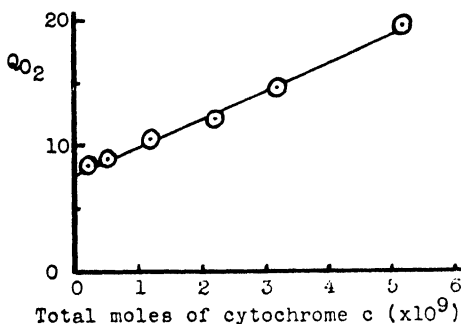


FIG. 3. Extrapolation to zero cytochrome concentration in the succinoxidase system. Each flask contained phosphate, succinate, and water as in Fig. 1, plus 0.3 ml. of 10 per cent liver homogenate containing  $2 \times 10^{-10}$  mole of cytochrome *c*, plus various amounts of added cytochrome to give the values indicated.

preceding one could also be explained on the basis of an alternate transport mechanism, the proposed explanation seems more in harmony with all of the facts. For instance, the fact that the oxygen uptake is completely inhibited by cyanide at a concentration between  $10^{-3}$  and  $10^{-4}$  M supports the idea that cytochrome *c* is an obligatory component of the system.

It is easy to prove that the activity of the succinoxidase system is bound up in particles which vary greatly in size. When a 10 per cent liver homogenate was centrifuged for 10 minutes at varying speeds and the supernatant fluid was assayed for succinoxidase in the presence of an excess of cytochrome *c*, it was found that over 50 per cent of the activity was sedimented at a speed of only

500 R.P.M. At a speed of 3500 R.P.M. over 90 per cent of the activity was sedimented. Further experiments with an air-driven centrifuge were made through the courtesy of Dr. H. Lundgren. After centrifuging at 23,000 R.P.M. for 40 minutes no succinoxidase could be detected in the supernatant by the Warburg technique. Only the smallest traces of succinic dehydrogenase were detectable by the spectrometric technique and it was shown that cytochrome oxidase was also present in similar amounts.

Experiments analogous to those listed up to this point were done under various conditions but those which have been reported were carried out under as nearly optimum conditions as possible, and were done subsequent to the following experiments which have to do with the selection of the buffer medium. It was found that Ringer-phosphate (calcium-free) gave much lower uptake than sodium phosphate ( $\text{NaH}_2\text{PO}_4$  plus  $\text{NaOH}$ ). The reason was traced to the chloride ion which is definitely inhibitory. The chloride effect is not due to hypertonicity. Inhibition by chloride is perhaps less surprising in view of the fact that in histochemical studies (10) chloride was not found inside the cells but was found only in the intercellular fluid. We found no essential difference between potassium phosphate and sodium phosphate. A pH of 7.4 was used because it is physiological and because it gave better rates than pH 7.0 or 8.2. The molarity of the phosphate could be increased or decreased by 50 per cent without affecting the  $Q_{O_2}$ , but larger changes gave marked decreases in the rate. Because of recent work by Colowick *et al.* (11, 12) magnesium ions plus or minus glucose were also tried as activators but no increase in rate was found. The homogenates apparently contain sufficient magnesium, phosphatase, and phosphate acceptor, so that these factors, if necessary, were not limiting. Since the experiments in this paper were completed, unpublished work by Axelrod, Swingle, and Elvehjem has shown that the calcium ion (10 to 50  $\gamma$ ) strongly activates the succinoxidase system in homogenates similar to those employed here. We have confirmed their observation at 38° but have found that at 24° the calcium effect is absent or negligible even with twice crystallized sodium succinate. That the calcium is effectively present in the experiments carried out at 24° is indicated by the relation between the rates with and without added calcium at the two temperatures.



*Effect of Acid Precipitation*

The usual method for the preparation of the succinoxidase system is to bring a phosphate extract of the tissue to a pH of 4.5 to

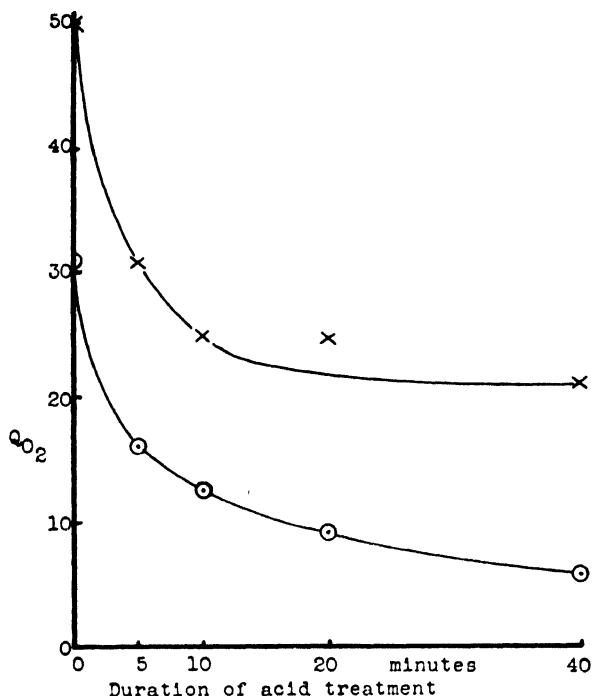


FIG. 4. The effect of acid precipitation (pH 4.6) on succinoxidase and cytochrome oxidase. Each flask contained phosphate and water as in Fig. 1, plus 0.5 ml. of  $10^{-4}$  M cytochrome *c* and 0.4 ml. of 10 per cent liver homogenate which had undergone various amounts of treatment, plus 0.3 ml. of 0.5 M succinate (indicated by  $\odot$ ) or 0.3 ml. of 0.1 M hydroquinone (indicated by  $\times$ ). The enzymes were precipitated by adding 0.5 ml. of 1 N acetic acid to 10 ml. of homogenate to give a pH of 4.6. The temperature was 22°. After the indicated periods of time 2 ml. portions were withdrawn from the whole and brought to pH 7.4 with 1 N NaOH. An equivalent amount of sodium acetate, pH 7.4, was added to the unprecipitated controls. The rate of autooxidation of hydroquinone was zero as determined in a mixture which was identical with the others except that the liver homogenate had been in a boiling water bath for 10 minutes, then rehomogenized before being used. The  $QO_2$  for hydroquinone is based on the first 10 minutes of oxygen uptake, since it is not linear.

4.6, at which point an abundant precipitate forms. This is centrifuged down and resuspended in phosphate for use (9, 13, 14). The homogenate used in the preceding section afforded an excellent opportunity to test the effect of acid treatment on the succinoxidase system. Fig. 4 shows that a marked loss in activity results from the acid precipitation and that both cytochrome oxidase and succinic dehydrogenase are damaged by the acid. The homogenization technique makes it possible to compare the activity of the partially "purified" product with the untreated preparation. A similar experiment carried out at 5° resulted in much less damage to the enzymes, as might be expected.

#### *Effect of Methylene Blue*

The effect of methylene blue on the succinoxidase system is of particular interest at the present in view of the newer knowledge concerning cytochrome *c* reduction (15). The fact that certain preparations can be obtained which will reduce methylene blue but not cytochrome *c* has been used as evidence that an extra carrier occurs between the dehydrogenase and cytochrome *c* (9, 16). Various reports in the literature have given conflicting information regarding the stimulatory or inhibitory effect of methylene blue on the succinoxidase system (5). A large number of experiments by the author have shown that the reports can all be explained on the basis of the simple fact that methylene blue inhibits a *complete* succinoxidase system and stimulates an *incomplete* succinoxidase system. This fact probably holds for the coenzyme systems as well, in which similar effects by methylene blue have been noted by the author (17). It is apparent that a succinoxidase system can be incomplete in a number of ways. Thus it can be deficient in cytochrome *c*, succinic dehydrogenase, or cytochrome oxidase. In Fig. 5, the upper curve demonstrates inhibition of a complete system, while the lower curve shows stimulation in a system in which the oxidase has been blocked by cyanide. The middle curve represents a system which is incomplete owing to cytochrome deficiency. Since the system contains a small amount of cytochrome, the curve is the resultant of both stimulation and inhibition. In Fig. 6 it is seen that methylene blue stimulates a succinoxidase system which is incomplete because of damage by acid

(probably denaturation) and also because of cytochrome deficiency. From these studies the conclusion emerges that data in the literature which show stimulation of the succinoxidase system by methylene blue are *prima facie* evidence that the system was incomplete. Since many studies have involved acid precipitation, incompleteness probably arose from damage due to acid as well as

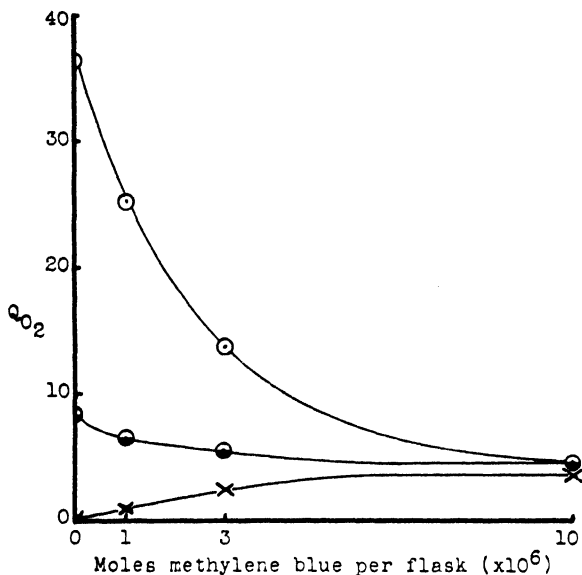


FIG. 5. The effect of methylene blue on various succinoxidase systems: complete, cytochrome-deficient, and with the oxidase blocked by cyanide. Each flask contained phosphate, succinate, and water as in Fig. 1 plus 0.3 ml. of 10 per cent liver homogenate and methylene blue as indicated. In addition the flasks contained cytochrome and cyanide as follows: ○, 0.3 ml. of  $10^{-4}$  M cytochrome per flask, no cyanide; ●, no cytochrome, no cyanide; ×, 0.3 ml. of neutral 0.01 M sodium cyanide. No alkali was present in the center cups.

from cytochrome deficiency. Spontaneous denaturation seems also to occur. From the coenzyme experiments (17) it seems likely that the same conclusion may apply to the coenzyme systems as well.

The inefficiency of methylene blue as a hydrogen carrier is also demonstrated in Fig. 5, from which it is seen that the addition of

methylene blue raised the  $Q_{O_2}$  of the cyanide-blocked system to a level slightly below that to which the complete system is lowered by the dye. From the data given it can be calculated that cytochrome *c* is about 1900 times more effective than methylene blue in this system.

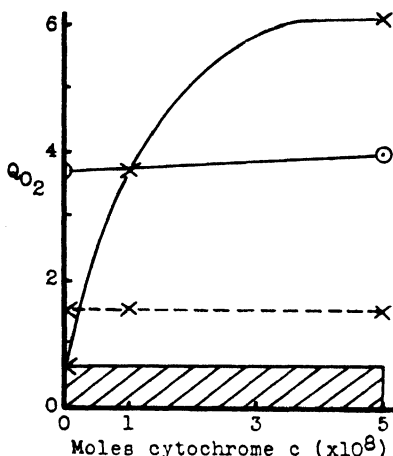


FIG. 6. The effects of methylene blue and cytochrome *c* on acid-precipitated succinoxidase. The effect of acid precipitation on the methylene blue reducing power. Each flask contained phosphate, succinate, and water as in Fig. 1, plus 1.0 ml. of 10 per cent liver homogenate, plus cytochrome as indicated.  $\times$  (continuous line), acid-precipitated enzyme (40 minutes at pH 4.6, see Fig. 4);  $\times$  (broken line), same as above, plus  $1 \times 10^{-5}$  mole of methylene blue (see Fig. 5);  $\circ$ , untreated enzyme plus sodium acetate as in Fig. 4 plus  $1 \times 10^{-5}$  mole of methylene blue. The  $Q_{O_2}$  of 1.0 ml. of untreated homogenate in a complete system is too rapid to measure but may be inferred from Figs. 1 and 2. The cross-hatched area indicates the extent of the endogenous respiration at this dilution as obtained from three separate controls.

#### DISCUSSION

It is apparent from the data presented that an intact cell is not a necessary condition for the effective functioning of the succinoxidase system. The possibility remains that there is a smaller architectural entity which is a functional unit in the system, and many of the properties seem to coincide with those which have been ascribed to mitochondria by Bensley (18). There is no

reason for believing that the succinoxidase system is unique in its ability to function in a disintegrated cell preparation which is properly supplemented. The main difference between this system and the coenzyme systems is that there are two more soluble biocatalysts in the latter, namely the dehydrogenase and the coenzyme, and both of these are undoubtedly limiting factors in the coenzyme systems in homogenates. Evidence is at hand that the link between the coenzyme and cytochrome *c* is closely comparable with succinic dehydrogenase and both are probably associated with the solid particles with which this study has dealt. There is reason to believe that both may be flavoproteins analogous to the cytochrome *c* reductase which has been obtained in soluble form from yeast by Haas, Horecker, and Hogness (15). Since the latter reacts directly with cytochrome *c*, it has been proved that no intermediate link is required. In view of the non-physiological character of methylene blue as compared with cytochrome *c* it seems undesirable to place too much emphasis on experiments in which the ability of an enzyme preparation to reduce cytochrome *c* has somehow been lost, while the ability to reduce methylene blue has been retained (9, 16). It is not impossible that the specific property of cytochrome *c* reduction may be lost by a denaturation process which leaves a portion of the non-specific dye-reducing property intact. This assumption deserves at least as much consideration as the assumption that a hypothetical third biocatalyst is an intermediary between the dehydrogenase and cytochrome *c*.

#### SUMMARY

1. The succinoxidase system was studied by use of a preparation of homogenized rat liver in high dilution, at which endogenous respiration was eliminated.

2. The liver homogenate oxidized succinate less rapidly than an equivalent weight of intact cells, as represented by a liver mince. This was considered to be evidence of a dilution effect. When the molarity of cytochrome *c* in the diluted homogenate was raised to the molarity of cytochrome *c* in the intact tissue, the dilution effect was eliminated and the  $Q_{O_2}$  was restored. Thus cytochrome *c* is probably the chief component which diffuses away from the system in the particles of the homogenate.

3. The usual method for preparing the succinoxidase system by precipitation at pH 4.6 results in the loss of most of the enzyme activity, on the basis of comparison with the original tissue.

4. The effect of methylene blue upon complete and incomplete succinoxidase systems was studied. It was shown that the dye inhibits a complete system and stimulates an incomplete system. The dye was shown to be about 1900 times less effective than cytochrome *c* as a hydrogen carrier in this system.

5. The results were discussed with reference to the mechanism of hydrogen transport in the succinoxidase system.

The author takes this opportunity to thank Professor T. R. Hogness of the University of Chicago for helpful discussion in connection with this problem.

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# THE DISSOCIATION OF CALCIUM AND MAGNESIUM CARBONATES AND BICARBONATES

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There is a steadily increasing amount of evidence that the salts of organic acids with calcium and with other bivalent cations behave as if incompletely dissociated in solution. This is particularly marked with di- and tribasic acids, but is also to be observed with monobasic acids (1-8). It is, therefore, quite likely that carbonic acid should form  $\text{CaHCO}_3^+$ ,  $\text{Ca}(\text{HCO}_3)_2$ , or  $\text{CaCO}_3$ , or any two, or all three, in solutions containing calcium.

Greenberg and Moberg (9) suggested the possibility of the formation of complexes of carbonate with calcium or magnesium or with other substances in order to account for the apparent low activity of the carbonic ion in sea water. The suggestion was rejected by Buch, Harvey, Wattenberg, and Gripenburg (10) and seems never to have been further tested.

The experiments to be reported were of two kinds: (1) titration of  $\text{H}_2\text{CO}_3$  in the presence of KCl and  $\text{CaCl}_2$  or  $\text{MgCl}_2$ ; (2) determination of the solubility of  $\text{CaCO}_3$  in KCl and in  $\text{KHCO}_3$  solutions.

## EXPERIMENTAL

*Titration Experiments*—Into 50 ml. volumetric flasks, there were measured known volumes of HCl and  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , or KCl solutions. After these were diluted to very nearly 50 ml., weighed amounts of  $\text{KHCO}_3$  were introduced, and the flasks stoppered and shaken until complete solution and thorough mixing had been obtained. If necessary,  $\text{H}_2\text{O}$  was added to the mark and the flasks were again inverted a few times. Alkaline mixtures were prepared by adding  $\text{KHCO}_3$  to the  $\text{MgCl}_2$  or  $\text{CaCl}_2$  solutions and then adding NaOH solution and diluting to the mark. The pH was then determined with the Coleman glass electrode assembly. The



composition of the mixtures and the results obtained in the presence of  $\text{MgCl}_2$  and of  $\text{CaCl}_2$  are given in Tables I and II. The results of the measurements in KCl solutions are not presented because they agreed with those calculated for  $\text{pK}'_1 = 6.34$  and  $\text{pK}'_2 = 10.10$ , taken from the data of Shedlovsky and MacInnes (11) and of MacInnes and Belcher (12).

*Solubility of  $\text{CaCO}_3$  in  $\text{KHCO}_3$  Solutions*—In all but two of Experiments 1 to 13, summarized in Table III, 25 mg. of a good grade of commercial c.p.  $\text{CaCO}_3$  were added to the 500 ml. of a solution containing known amounts of KCl,  $\text{KHCO}_3$ , and HCl, with a total ionic concentration of 0.152. These solutions were contained in glass-stoppered volumetric flasks. In one experiment 50 mg. and in another 250 mg. of  $\text{CaCO}_3$  were added as a check on the purity of the substance. After the solutions had stood at room temperature for the number of days indicated, with occasional shaking, pH was determined with a glass electrode and the remainder of the solution was filtered through paper. The first fifth was discarded and portions of the remainder were used for the determination of calcium by oxalate precipitation and permanganate titration. With small concentrations of calcium, as much as 100 ml. of solution was acidified and evaporated to 15 ml. before precipitation. The total  $\text{CO}_2$  content was computed from the amount of bicarbonate added. In the case of the solutions not containing any bicarbonate,  $[\text{CO}_3^{=}]$  was calculated from pH and  $[\text{Ca}^{++}]$  in the following manner, suggested by Dr. R. K. Cannan.

Since

$$2[\text{Ca}^{++}] + [\text{H}^+] = 2[\text{CO}_3^{=}] + [\text{HCO}_3^-] + [\text{OH}^-]$$

and since  $[\text{H}^+]$  and  $[\text{H}_2\text{CO}_3]$  were negligible under the conditions employed, we may write

$$2[\text{Ca}^{++}] = [\text{CO}_3^{=}] + \frac{K'_2 + [\text{H}^+]}{K'_2} [\text{CO}_3^{=}] + [\text{OH}^-]$$

from which

$$[\text{CO}_3^{=}] = (2[\text{Ca}^{++}] - [\text{OH}^-]) \frac{K'_2}{2K'_2 + [\text{H}^+]}$$

## DISCUSSION

*Titration Experiments*—As may be seen from Table I, the pH of the mixtures of  $\text{KHCO}_3$  and  $\text{MgCl}_2$  varied from 7.70 to 7.75, instead of being 8.22, as calculated from the dissociation constants. Mixtures containing added acid or alkali were, similarly, more acid than those in which KCl was used instead of  $\text{MgCl}_2$ . Attempts were made to formulate these differences as being due to the formation of  $\text{Mg}(\text{HCO}_3)_2$ ,  $\text{MgCO}_3$ , and  $\text{MgHCO}_3^+$ . The first was obviously unsatisfactory and the other two gave values for the dissociation constants that varied, regularly, with the pH. This indicated the possibility that both were formed and that the relative amount varied with the pH.

If we let  $B = 1$  minus the equivalents of HCl or plus the equivalents of NaOH added in the presence of  $\text{MgCl}_2$ ,<sup>1</sup>  $b = 1$  minus the equivalents of HCl or plus the equivalents of NaOH per mole of total  $\text{CO}_2$  added in the presence of KCl of the same ionic strength and at the same pH.

$$C = \text{moles } \text{MgHCO}_3^+$$

$$K'_4 = \frac{[\text{H}^+][\text{MgCO}_3]}{[\text{MgHCO}_3^+]}$$

$$\text{and } A = K'_4/[\text{H}^+]$$

$$\text{then } AC = \text{moles } \text{MgCO}_3$$

$$\text{and } B = b(1 - C - AC) + C + 2AC, \text{ from which,}$$

$$C = \frac{B - b}{(1 + 2A) - b(1 + A)}$$

If we assume a value of  $K'_4$  and let  $\text{p}a\text{H}^+ = \text{p}[\text{H}^+]$ , it is possible to calculate  $C$ , and from this, and the values for  $[\text{total } \text{CO}_2]$ ,  $K'_1$ ,  $K'_2$ ,  $B$ , and  $[\text{Mg}^{++}]$ , the value of  $K'_{\text{diss.}} = [\text{Mg}^{++}][\text{HCO}_3^-]/[\text{MgHCO}_3^+]$ . As may be seen from Table I, the assumption that  $\text{p}K'_4 = 8.50$  leads to values for  $K'_{\text{diss.}}$  ranging from 0.11 to 0.26. The two values that fall outside this range were obtained under conditions leading to the formation of very small amounts of complex. The twenty-one acceptable determinations led to an average value for  $K'_{\text{diss.}} = 0.17 \pm 0.01$ ,  $\text{p}K'_{\text{diss.}} =$

<sup>1</sup> At the pH of these experiments, the water correction was negligible.



$0.77 \pm 0.02$ . Actually the values are not known as accurately as these figures would indicate, for a change in the value of  $pK'_4$  to 8.40 makes  $K'_{\text{diss.}} = 0.21 \pm 0.01$ .

The lesser solubility of  $\text{CaCO}_3$  restricted the range of observations to be made in the presence of  $\text{CaCl}_2$  to the region in which complex formation is less marked. However, it was evident that mixtures containing  $\text{CaCl}_2$  were even more acid than were those containing  $\text{MgCl}_2$  in the same concentration (Table II). In terms of our theory, this might be due to a greater tendency to complex formation or to a greater dissociation of  $\text{H}^+$  from the former than from the latter. Both possibilities were explored and it was found that the more consistent values were obtained by assuming that  $pK'_4$  for  $\text{CaHCO}_3^+ = 7.90$ . With this value, it was found that  $K'_{\text{diss.}} \text{CaHCO}_3^+$  varied from 0.12 to 0.35. Except for one value, obtained under conditions under which very little complex was formed, the average was  $0.16 \pm 0.01$ ;  $pK' = 0.80 \pm 0.03$ . Just as with  $K'_{\text{diss.}} \text{MgHCO}_3^+$ , a change in the value of  $pK'_4$  to 7.80 produces a change in the value of  $K'_{\text{diss.}} \text{CaHCO}_3^+$ . With this assumption, the value becomes  $0.19 \pm 0.02$ .

From the values for  $pK'_{\text{diss.}} \text{MHCO}_3^+$  and  $K'_4$  thus obtained, it is possible to calculate values for  $pK'_{\text{diss.}} \text{MCO}_3$  as follows:

$$\begin{aligned} pK'_{\text{diss.}} \text{MHCO}_3^+ &= p[\text{M}^{++}] + p[\text{HCO}_3^-] - p[\text{MHCO}_3^+] \\ pK'_4 &= \text{pH} + p[\text{MCO}_3] - p[\text{MHCO}_3^+] \\ pK'_{\text{diss.}} \text{MHCO}_3^+ - pK'_4 &= p[\text{M}^{++}] + p[\text{HCO}_3^-] - \text{pH} - p[\text{MCO}_3] \\ pK'_2 &= -p[\text{HCO}_3^-] + \text{pH} + p[\text{CO}_3^{--}] \\ pK'_{\text{diss.}} \text{MHCO}_3^+ - pK'_4 + pK'_2 &= p[\text{M}^{++}] + p[\text{CO}_3^{--}] - p[\text{MCO}_3] \\ &= pK'_{\text{diss.}} \text{MCO}_3 \end{aligned}$$

The value for  $pK'_{\text{diss.}} \text{MgCO}_3$  thus obtained is 2.37, which may be compared with the value for  $\text{Mg}$  oxalate, 2.55 at  $\mu = 0.2$  and 3.43 at  $\mu = 0$ , found by Cannan and Kibrick (2) and by Money and Davies (3) respectively. Similarly the value for  $pK'_{\text{diss.}} \text{CaCO}_3$  is 3.00, which is precisely that found by Money and Davies for  $\text{Ca}$  oxalate at  $\mu = 0$ . The value 5.33 previously reported by Greenwald (1) for  $\text{CaC}_2\text{O}_4$  may be too high, perhaps because mixed complexes of  $\text{Ca}$  oxalate and citrate were formed.

*Solubility of  $\text{CaCO}_3$* —The value for the negative logarithm of the average value of the solubility product  $[\text{Ca}^{++}][\text{CO}_3^{--}]$  in  $\text{KCl}$  solution was 8.007. It was less than this in solutions containing

TABLE II  
*Titration of  $\text{KHCO}_3$  in Presence of  $\text{CaCl}_2$ , at Approximately  $22^\circ$*

Total CO <sub>2</sub> ..... " Ca		0.0152 0.0453				0.0304 0.0444				0.0608 0.0348						
HCl × 10 <sup>3</sup>		pH	CaHCO <sub>3</sub> <sup>+</sup> × 10 <sup>3</sup>	CaCO <sub>3</sub> × 10 <sup>3</sup>	K* × 10	μ	pH	CaHCO <sub>3</sub> <sup>+</sup> × 10 <sup>3</sup>	CaCO <sub>3</sub> × 10 <sup>3</sup>	K* × 10	μ	pH	CaHCO <sub>3</sub> <sup>+</sup> × 10 <sup>3</sup>	CaCO <sub>3</sub> × 10 <sup>3</sup>	K* × 10	μ
15.89							6.20	2.83	0.06	1.7	0.156					
13.90							6.53	4.44	0.19	1.4	0.152	6.80	4.92	0.40	2.3	0.149
9.93							6.80	5.26	0.42	1.4	0.149	6.92	8.17	0.86	1.3	0.141
5.96												7.10	9.06	1.44	1.2	0.138
3.97							6.98	5.17	0.62	1.5	0.149	7.25	7.49	1.68	1.6	0.142
1.99		7.05	1.49	0.21	3.5†	0.156	7.27	3.70	0.87	2.5	0.152					
0.00		7.40	2.83	0.89	1.7	0.150	7.42	5.29	1.75	1.6	0.148					

Average  $K = [\text{Ca}^{++}][\text{HCO}_3^-]/[\text{CaHCO}_3^+] = 0.16 \pm 0.01$ ;  $\text{pK} = 0.80 \pm 0.03$ .

\* Assuming  $\text{pK}_a = -\log ([\text{H}^+][\text{CaCO}_3]/[\text{CaHCO}_3^+]) = 7.90$ .

† Excluded from the average.

large amounts of  $\text{KHCO}_3$ , indicating complex formation. The extent of this was calculated as follows:

$$\begin{aligned} p[\text{Ca}^{++}] &= 8.007 - p[\text{CO}_3^{--}] \\ [\text{Total complex}] &= [\text{total Ca}] - [\text{Ca}^{++}] \end{aligned}$$

$$[\text{CaHCO}_3^+] = \frac{[\text{total complex}]}{1 + (K' / [\text{H}^+])}$$

$K' = [\text{Ca}^{++}][\text{HCO}_3^-] / [\text{CaHCO}_3^+]$  was then calculated in the usual manner.

TABLE III  
*Solubility of  $\text{CaCO}_3$  at Approximately  $22^\circ$  and  $\mu$  0.152*

Experiment No.	Days	Total $\text{CO}_2$	$\text{HCl} \times 10^3$	pH	Total Ca $\times 10^3$	Apparent $pK_{s.p.} \text{CaCO}_3$	Assuming $pK_{s.p.} = 8.007$ and $pK_4 = 7.90$	
							$\text{CaHCO}_3^+ \times 10^3$	$\frac{[\text{Ca}^{++}][\text{HCO}_3^-]}{[\text{CaHCO}_3^+]} \times 10$
1	21		0.1	8.85	28.5	8.09		
2*	21		0.2	8.50	45.5	8.01		
3	11			9.53	17.6	8.01		
4	11			9.50	19.5	7.93		
5†	3			9.30	21.0	8.00		
Average...						8.007		
6	18	0.152	4.86	7.90	3.28	7.32	1.30	0.76
7	18	0.122	4.86	7.80	4.25	7.61	1.44	1.37
8	18	0.0912	4.86	7.67	5.53	7.75	1.56	1.70
9	18	0.0608	4.86	7.40	11.50	7.89	2.03	1.42
10	120	0.152	9.72	7.70	7.06	7.39	3.28	0.75
11	120	0.122	7.78	7.58	8.89	7.51	4.09	0.80
12	120	0.0912	5.83	7.70	8.84	7.51	3.68	0.67
13	120	0.0608	3.89	7.52	9.29	7.86	2.17	1.72

Average  $K' = [\text{Ca}^{++}][\text{HCO}_3^-] / [\text{CaHCO}_3^+] = 0.15 \pm 0.02$ ;  $pK = 0.82 \pm 0.06$ .

\* 50 mg. of  $\text{CaCO}_3$ .

† 250 mg. of  $\text{CaCO}_3$ .

The average for all eight determinations was  $0.15 \pm 0.02$ ,  $pK' = 0.82 \pm 0.06$  (Table III). Considering the numerous errors involved, these values are remarkably close to the value found in the titration experiments.

*Physiological Significance*—From the figures presented in this paper, it would appear that ultrafiltrates of plasma or serum containing 1.25 mm of total Ca and 20 mm of  $\text{BHCO}_3$  must contain about 0.14 mm of  $\text{CaHCO}_3^+$  and 0.04 mm of  $\text{CaCO}_3$ . It would appear, therefore, that the frog heart method of McLean and Hastings (13) may measure not  $[\text{Ca}^{++}]$  but calcium available to the frog heart. In simple saline-bicarbonate solution, this may be  $\text{Ca}^{++}$ ,  $\text{CaHCO}_3^+$ , or  $\text{CaCO}_3$ , or all of them. In serum or plasma, or their ultrafiltrates, it may include other complexes of calcium.

## SUMMARY

In the presence of  $\text{CaCl}_2$  or of  $\text{MgCl}_2$ , carbonic acid appears to have a greater  $\text{H}^+$  dissociation than in KCl solutions of similar ionic strength. The solubility product  $[\text{Ca}^{++}][\text{CO}_3^{--}]$  appears to be greater in  $\text{KHCO}_3$  solution than in KCl solutions. These differences are believed to be due to the formation of complexes of the types  $\text{MHCO}_3^+$  and  $\text{MCO}_3$ . There have been calculated values for

$$-\log \frac{[\text{H}^+][\text{MCO}_3]}{[\text{MHCO}_3^+]} \quad \text{and} \quad -\log \frac{[\text{M}^{++}][\text{HCO}_3]}{[\text{MHCO}_3^+]}$$

that are quite consistent over great variations in pH, concentrations of total  $\text{CO}_2$ , and ratio of Ca, or Mg, to  $\text{CO}_2$ .

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## CARBON SUBOXIDE AND PROTEINS

### IV. THE ULTRACENTRIFUGAL BEHAVIOR OF CARBON SUBOXIDE-TREATED HORSE SERUM ALBUMIN\*

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In the reaction between carbon suboxide,  $O=C=C=C=O$ , and proteins, already described (1), there was postulated the existence of intermediates of the type  $O=C=CH-CO-NH$ -protein or  $O=C=CH-CO-O-C_6H_4$ -protein from reaction with the amino groups of lysine or the phenolic hydroxyls of tyrosine, respectively. The residual ketene linkage must react further either with a molecule of water to form a malonyl derivative with a free carboxyl group or with another amino or phenolic group to form a diamide, diester, or mixed derivative. In the earlier work it was found that the reaction with water was preponderant, thus replacing original amino or phenolic groups with carboxyls.

The second possibility was not, however, eliminated. Reaction of the intermediate with additional side chain residues might be *intramolecular* if appropriate groups were sufficiently near the reactive ketene linkage on the same protein surface, or the reaction might be *intermolecular* if an amino or phenolic group of another protein molecule approached the ketene linkage first. Intermolecular reaction of this kind would result in the coupling of protein molecules to give products with twice the molecular size of the starting material, and further reaction of the dimer might lead to a trimer,<sup>1</sup> the probability of such extended reactions decreasing with the complexity of the molecules, however.

\* This investigation has been supported in part by grants from the Rockefeller Foundation.

<sup>1</sup> The terms dimer and trimer are freely used here to indicate the products formed by the coupling of 2 or 3 protein molecules by malonyl bridges. Polymerization refers to this process.



An ultracentrifugal examination of carbon suboxide-treated horse serum albumin has been made to determine the size distribution of the treated protein. Fig. 1, *B* shows the sedimentation diagram obtained in the ultracentrifuge for a normal lot of the malonyl protein, and indicates that the bulk (about 84 per cent) of this material has a sedimentation constant  $s_{20,w}^{1\%} = 4.5 \times 10^{-13}$ . The presence of faster sedimenting materials is also indicated, with about 12 per cent of the total protein having a sedimentation constant  $s_{20,w}^{1\%} = 6.6 \times 10^{-13}$ , and about 4 per cent having about  $8 \times 10^{-13}$ .

The absence of these faster sedimenting substances in the starting material is shown by Fig. 1, *A*, the sedimentation diagram of the native protein used. The observed sedimentation constant for this material was  $s_{20,w}^{1\%} = 4.1 \times 10^{-13}$ .

An ammonium sulfate fractionation of malonyl serum albumin was undertaken in order to concentrate the faster sedimenting components. It was found that the concentration of these components was increased in the first fraction to precipitate (below 1.8 M  $(\text{NH}_4)_2\text{SO}_4$ ), the sedimentation diagram of this fraction being illustrated in Fig. 1, *C*. This fraction, representing 36 per cent of the total protein nitrogen, contained approximately 55 per cent of materials of sedimentation constant  $s_{20,w}^{1\%} = 4.5 \times 10^{-13}$ , 30 per cent of  $6.6 \times 10^{-13}$ , and 15 per cent of  $8.5 \times 10^{-13}$ . In subsequent fractions at higher ammonium sulfate concentrations, the amount of faster sedimenting substances was greatly diminished.

The higher sedimentation constants observed here cannot readily be explained by a more symmetrical molecule of 70,000 to 80,000 molecular weight,<sup>2</sup> since the sedimentation constant for a spherical molecule of this weight (the maximum possible value) would be only  $5.6$  to  $6.2 \times 10^{-13}$ , unless the partial specific volume was considerably different from 0.75. Without additional data for the frictional ratio,  $f/f_0$ , and partial specific volume,  $\bar{v}$ , it is not possible to calculate their molecular weights. In the absence of data for  $f/f_0$ , we may consider various possible interpretations. If we assume the frictional ratio  $f/f_0 = 1.37$  and  $\bar{v} = 0.748$ , as obtained for normal serum albumin,<sup>3</sup> we find that the sedimenta-

<sup>2</sup> The molecular weight of the malonyl serum albumin monomer would be about 10 per cent greater than the untreated serum albumin because of the weight of added malonyl groups. (See the experimental section.)

<sup>3</sup> Mehl, J. W., and Oncley, J. L., unpublished observations.

tion constants  $4.5$ ,  $6.9$ , and  $8.5 \times 10^{-13}$  correspond to molecular weights of  $80,000$ ,  $150,000$ , and  $210,000$ , respectively, or approximately 1, 2, and 3 times the molecular weight of native horse serum albumin. This assumption of similar values for the frictional ratios of all these molecules would seem to be unjustified, however. Another possible interpretation is that both sedimentation constants,  $6.9$  and  $8.5 \times 10^{-13}$ , represent molecules of about

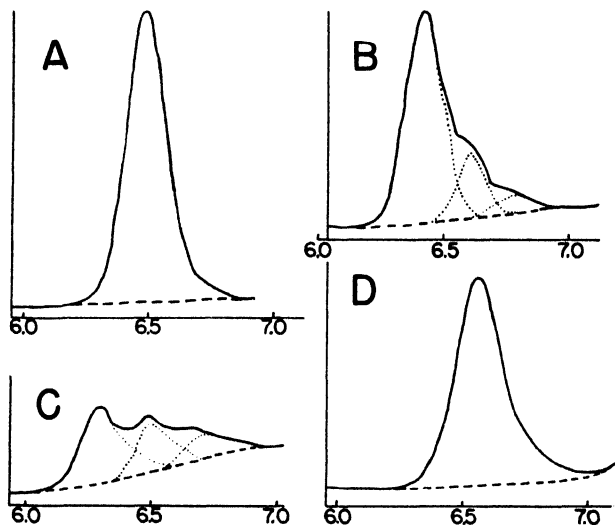


FIG. 1. Ultracentrifuge sedimentation diagrams. The ordinates represent refractive index gradient; the abscissas, distance from the center of rotation (in cm.). The time after reaching full speed (in minutes) =  $t$ . *A* represents native horse serum albumin,  $t = 100$ ; *B*, unfractionated carbon suboxide-treated serum albumin, Preparation CSA-7,  $t = 60$ ; *C*, fractionated carbon suboxide-treated serum albumin, Preparation CSA-6, Fraction A,  $t = 60$ ; *D*, carbon dioxide-treated serum albumin, Preparation CO<sub>2</sub>SA-2,  $t = 100$ . These diagrams are tracings of the schlieren photographs.

$160,000$  molecular weight. We then would obtain  $f/f_0$  values of  $1.41$  and  $1.15$ , respectively, for these molecules if we continue to assume  $\bar{v} = 0.748$ . The most probable values for the axial ratio,  $a/b$ , and the hydration,  $w$ , of normal serum albumin may be taken as  $a/b = 5$  and  $w = 0.2$  gm. of water per gm. of dry protein (2). If we assume the hydration to be unchanged, then the  $f/f_0$  values just obtained give us axial ratios of  $a/b = 6$  and  $2.5$ , respectively, which are reasonable ratios for possible dimer molecules.

The possibility that incipient denaturation, which may have occurred during the preparation of malonyl serum albumin, is partly responsible for the inhomogeneity of the product has been considered. That the malonyl protein is freely soluble in water at its isoelectric point indicates that little denaturation has taken place. To clarify this point further serum albumin was treated with  $\text{CO}_2$  under conditions parallel to those used in the  $\text{C}_3\text{O}_2$  reaction. The pH, however, was 9.5 instead of 7.5, and the reaction period was extended from 1 to 2 hours (Table I, Preparation  $\text{CO}_2\text{SA-2}$ ). Even under these conditions no indication of polymerization was observed (Fig. 1, *D*). An experiment at pH 8.0 for 2 hours was likewise negative (Preparation  $\text{CO}_2\text{SA-1}$ ).

The existence of polymerized protein molecules would be in agreement with the interpretation previously adopted for the carbon suboxide reaction (1). The dimer having a molecular weight of about 160,000 would be analogous to malonyldiglycine, the compound obtained from the reaction of carbon suboxide with glycine. The trimer, if present, would result from a further reaction of the dimer.

#### EXPERIMENTAL

*Carbon Suboxide Horse Serum Albumin (Preparation CSA-6)*—Carbohydrate-free crystalline horse serum albumin was treated at pH 7.5 with 0.4 mole of carbon suboxide per gm. atom of nitrogen over a period of 1 hour (1). The purified product had an amino to total nitrogen ratio of 0.008 and contained 0.080 mole of malonic acid per gm. atom of nitrogen.

*Preparation CSA-7*—This was a similar preparation in which the amino to total nitrogen ratio was 0.004 and the content of malonic acid per gm. atom of nitrogen 0.099.

*Fractionation of  $\text{C}_3\text{O}_2$  Horse Serum Albumin*—The apparatus shown in Fig. 2 has been found satisfactory for the diffusion of salt across a cellophane membrane into small volumes of protein solution. The membrane was held between the openings of the flasks *A* and *B* by rubber washers and clamps fitted over the ring necks. The smaller flask of 30 ml. capacity, into which extended a suitable stirring device, was filled with the protein solution, and ammonium sulfate was dialyzed in from the larger 225 ml. flask.

With this apparatus, ammonium sulfate solution of pH 5.9 was dialyzed against malonyl serum albumin Preparation CSA-6 to give four fractions,<sup>4</sup> as follows: Fraction A, 0 to 1.8 M in  $(\text{NH}_4)_2\text{SO}_4$  containing 36 per cent of the protein nitrogen; Fraction B, 1.9 to 2.2 M, 35 per cent; Fraction C, 2.2 to 2.5 M, 17 per cent; Fraction D, 2.5 to 4.1 M, 6 per cent. These fractions were purified by dialysis and prepared for the ultracentrifuge as described below.

*Carbon Dioxide Treatment of Native Serum Albumin*—The data for the preparations are presented in Table I. The experimental procedure was the same as that in the  $\text{C}_3\text{O}_2$  reaction.

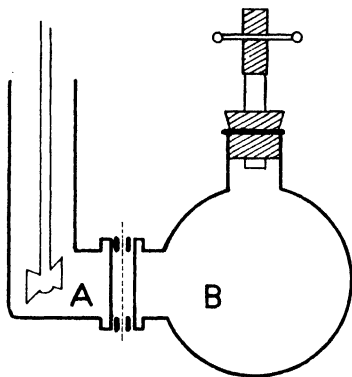


FIG. 2. Apparatus for small scale dialysis experiments (see the text)

*Ultracentrifuge Experiments*—The ultracentrifuge, of the air-driven type (3), was equipped with an optical system of the Philpot schlieren type (4), and was run at a speed of 54,000 R.P.M. with a cell 15 mm. high and a solution thickness of 10 mm. A General Electric type H-6 mercury arc lamp, unfiltered, was the light source, and Eastman Contrast Process plates were used for photographing the sedimentation diagrams. 1 per cent protein solu-

<sup>4</sup> The first carbon suboxide-treated serum albumin preparation to be studied in the ultracentrifuge, and the fractionation of this material, gave results which were not in agreement with those recorded here, the sedimentation constants observed for these solutions always being considerably smaller, and the apparent diffusion constants (as measured by the width of the sedimenting boundary) being smaller also. The interpretation of these earlier results is not clear. All later results have yielded consistent data in good agreement with those recorded here.

tions in 0.2 M potassium chloride were employed for the ultracentrifuge analyses. Since the addition of potassium chloride often caused the solution to become slightly cloudy, approximately 0.1 ml. of 0.5 N potassium hydroxide was added to bring the pH up to 5.2 from 4.6, the point of minimum solubility of malonyl serum albumin. The sedimentation constants,  $s_{20,w}^{1\%}$ , of these solutions have been corrected to the density and viscosity of water at 20° (assuming a partial specific volume,  $\bar{v}$ , of 0.748), but *not* to zero protein concentration.

TABLE I  
*Carbon Dioxide Treatment of Native Serum Albumin*

Preparation No.....	CSA-7	CO <sub>2</sub> SA-1	CO <sub>2</sub> SA-2
Protein, gm.....	2.0	2.0	0.5
Initial volume, ml.....	32.0	32.0	20.0
Length of reaction, hrs.....	1.0	2.0	2.0
pH.....	7.5	8.0	9.5
Gas added, ml.....	218	440	440

#### SUMMARY

The product from the reaction of carbon suboxide with horse serum albumin has been found by ultracentrifugal analysis to contain an appreciable proportion of materials sedimenting faster than normal serum albumin.

This faster sedimenting material, consisting of two discrete components, can be interpreted as representing a dimer of somewhat elongated shape and either an almost symmetrical dimer or an elongated trimer. This interpretation of the reaction of carbon suboxide with proteins is in agreement with mechanisms already postulated.

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# THE SYNTHESIS OF NICOTINIC ACID BY THE RAT

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Attempts to demonstrate the synthesis of nicotinic acid by rats subsisting on a diet containing little of this factor have provided suggestive evidence that such synthesis does occur. Shourie and Swaminathan (1) performed balance experiments in which the nicotinic acid intake and excretion were measured and they reported an excess of excretion over intake, but their findings are open to criticism on account of the method used by them for estimating nicotinic acid. Charcoal was used in treating urine hydrolysates and extracts of solid matter, and the extracts were not fully decolorized before the König reaction was applied; each of these points means the introduction of serious error (2, 3). Another type of evidence was produced by Dann and Kohn (4), who found that rats growing on a diet low in nicotinic acid synthesized and laid down in their tissues V factor (coenzymes I and II) containing more nicotinic acid than was being ingested. In this paper new evidence will be presented which is based on the increase of nicotinic acid in the tissues of rats growing quite rapidly on diets very low in nicotinic acid. This increase has been demonstrated by direct analysis of the tissues for nicotinic acid and the results are therefore not open to the objection which may be urged against those based on V factor measurements; namely, that V factor in the body of the rat might have been increased at the expense of nicotinic acid which was initially present.

*Experiments on Synthesis*—Jukes (5) has reported raising and breeding rats on a diet in which the vitamin B complex was provided in the form of synthetic thiamine chloride, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, and choline chloride. For the experiments described below nicotinic acid was omitted

from the diet of Jukes and other minor modifications were made so that our basal diet (Diet S-1) was composed as follows: Labco casein 1000 gm., sucrose 2400 gm., cottonseed oil 320 gm., B. D. H. salts<sup>1</sup> 280 gm., choline chloride 2 gm., thiamine chloride 10 mg., riboflavin 20 mg., pyridoxine 10 mg., calcium pantothenate 120 mg., and 2-methyl-1,4-naphthohydroquinone diacetate 5 mg. This diet was supplemented daily for each rat with 1 drop of a mixture of equal volumes of cod liver oil and wheat germ oil given by pipette. The casein was the only component containing nicotinic acid; it contained 2.1  $\gamma$  per gm.; therefore the complete diet contained 0.525  $\gamma$  of nicotinic acid per gm.

In a preliminary experiment twenty weanling rats of the Vanderbilt strain (7) were put on Diet S-1 when 25 days old, with an average weight of 46 gm. Both sexes were represented and there were nine pairs of litter mates matched in sex and weight. The rats were divided into two groups, of which those in Group 1 received only water and the basal diet, while those in Group 2 each received in addition 1 mg. of nicotinic acid daily in solution from a dropping pipette. During the course of the experiment three rats died following lung infections, one from Group 2 and two from Group 1. Eight of the matched pairs survived the experimental period of 63 days. During this period there was no significant difference in the weight increases of the two groups, and all of the survivors appeared to be vigorous and healthy.

Five of the rats which had received Diet S-1 without added nicotinic acid were killed for nicotinic acid determinations on liver, kidney cortex, and muscle. The levels observed did not differ significantly from those reported for adult rats of this strain raised on a stock diet (3). The mean nicotinic acid content of the whole liver of these rats was 1290  $\gamma$ , compared with 410  $\gamma$  in the whole livers of weanlings (average for eight rats). Thus the mean increase in nicotinic acid content of the whole liver of these rats during 63 days on Diet S-1 was close to 880  $\gamma$ . Measurement of the food intake of the rats showed that the average ingestion of nicotinic acid in the diet over the whole 63 day period was 550  $\gamma$ . Hence the evidence of the liver strongly suggested that these rats had been synthesizing nicotinic acid.

<sup>1</sup> Made to the formula of Drummond and Watson (6).

A further experiment was then made in order to establish beyond reasonable doubt that synthesis occurs on this diet and to gain some idea of the amount of nicotinic acid synthesized. From four litters of rats weaned at 21 days the males were segregated and given a stock diet. When 25 to 28 days old, litter mate pairs of closely similar weight were chosen and one from each pair assigned to each of two groups. All the rats in Group 1 were killed at once for estimation of the total nicotinic acid content of the body as described below. Those in Group 2 were housed in separate cages and given Diet S-1 and water; the food intake was followed daily. After 4 weeks they were killed for the estimation of the body nicotinic acid.

In order to estimate its total nicotinic acid content, the rat was killed by a blow on the head and immersed in a 10 per cent BaS suspension to remove the hair. The carcass was rinsed in water and dried and then passed five or six times through a meat grinder, until the minced tissue appeared to be thoroughly mixed. From 15 to 30 gm. of the tissue were then weighed and transferred to a Waring blender<sup>2</sup> with the required volume of water to make a final volume of 250 ml. The tissue was homogenized and the suspension allowed to stand for 5 minutes in order to permit the dispersed air to separate from it. 10 ml. aliquots of the suspension were taken for digestion with HCl and determination of the nicotinic acid as already described (3). From the nicotinic acid content of the aliquot the total nicotinic acid content of the rat was calculated. The data, together with the nicotinic acid intake of each rat fed Diet S-1, are collected in Table I. Assuming that at the beginning of the experiment each rat in Group 2 had a total body nicotinic acid content equal to that of its litter mate in Group 1, then synthesis of nicotinic acid had occurred at least to the amount given in the last column of Table I.

*Site of Synthesis*—Although the observations described above gave clear evidence that the rats synthesized nicotinic acid when fed Diet S-1, they provided no clue to the place where the synthesis occurred. The most immediate question here was whether the synthesis was carried out by microorganisms in the intestinal canal or occurred within the tissues of the rat. In order to obtain

<sup>2</sup> Obtained from the Fisher Scientific Company, Pittsburgh, Pennsylvania.



information on this question an experiment was arranged in which sulfaguanidine was added to Diet S-1 as a means of decreasing

TABLE I

*Demonstration of Synthesis of Nicotinic Acid by the Rat*

Each pair of rats on the same line is a litter mate pair. The rat in Group 1 was killed at weaning for determination of the total body nicotinic acid content. The rat in Group 2 was maintained on Diet S-1 for 4 weeks and then treated similarly.

Group 1		Group 2			
Rat No.	Nicotinic acid in body at weaning	Rat No.	Nicotinic acid in body after 28 days on Diet S-1	Nicotinic acid ingested in 28 days	Nicotinic acid synthesized in body
	mg.		mg.	mg.	mg.
1	2.7	1	8.0	0.14	5.2
2	2.5	2	7.3	0.13	4.7
3	2.6	3	7.2	0.14	4.5
4	2.6	4	7.8	0.13	5.1
5	2.5	5	8.7	0.15	6.0
6	2.2	6	7.1	0.12	4.8
7	2.1	7	6.8	0.11	4.6
8	1.4	8	6.3	0.10	4.8
Mean . . . . .	2.3		7.4	0.13	5.0

TABLE II

*Effect on Rat Growth of Addition of 1 Per Cent Sulfaguanidine or 1 Per Cent Sulfaguanidine + 1 Mg. of Nicotinic Acid Daily to Diet S-1*

	Group A	Group B	Group C
Diet . . . . .	S-1	S-1 + 1% sulfaguani- dine	S-1 + 1% sulfaguani- dine
Nicotinic acid supplement per day, mg. . . . .	None	None	1
Mean weight increase after 42 days, gm. . . . .	164	84	79

greatly the numbers of intestinal bacteria and therefore also the amount of nicotinic acid which could be produced by them.

A test experiment showed that the addition of sulfaguanidine

to Diet S-1 caused a decrease in the rate of growth of rats on this diet. This might have been due to a suppression of intestinal synthesis of nicotinic acid, or to some other cause such as a direct toxic action of the drug on the rat. To decide between these possibilities the following experiment was performed. Young male rats 25 days old were divided into three groups, so that each group contained one rat from each of ten trios of matched litter mates, and the rats were individually housed. Group A received Diet S-1; Groups B and C received Diet S-1 with 1 per cent of added sulfaguanidine. Each rat in Group C was given in addition a daily dose of 1 mg. of nicotinic acid in solution from a pipette. The results of the experiment are shown in Table II. The effect of the sulfaguanidine was to decrease the rate of growth, but the addition of nicotinic acid at the same time did not counteract the effect of the sulfaguanidine.

From this result it was concluded that the effect of sulfaguanidine was not due to a suppression of intestinal synthesis of nicotinic acid; and since the drug largely decreased the number of intestinal microorganisms, it was also concluded that no important part of the synthesis of nicotinic acid by rats on Diet S-1 took place in the intestine.

#### SUMMARY

It has been shown by direct measurement of the body nicotinic acid of rats growing on a diet low in nicotinic acid that the rats were able to synthesize nicotinic acid. During the 2nd month of life, while they ingested about 5  $\gamma$  of nicotinic acid daily, the body content increased daily by an average amount of the order of 200  $\gamma$ . No measurements of nicotinic acid in the urine and feces were made, but it has been shown by Perlzweig and Huff<sup>3</sup> that excretion of nicotinic acid by the rat continues even when none is ingested; therefore the figures for body increase less ingestion represent a minimum figure for the amount of synthesis.

The evidence obtained suggests that the place of synthesis is within the body of the rat, and that it is not due to the symbiotic activity of microorganisms in the intestine.

This evidence appears to leave no doubt that nicotinic acid is not a vitamin for the rat, as has been suggested in the past (8).

<sup>3</sup> Huff, J., and Perlzweig, W. A., *J. Biol. Chem.*, in press.

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# THE SYNTHESIS AND BREAKDOWN OF LIVER PHOSPHOLIPID IN VITRO WITH RADIOACTIVE PHOSPHORUS AS INDICATOR\*

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The conversion of phosphate marked by the inclusion of radioactive phosphorus to labeled phospholipid has been used to compare phospholipid activities of various tissues in the intact animal (1-4). Although it has been suggested that tissues with higher activities possess the power of synthesizing phospholipid, it is recognized that in a complex system such as the intact animal it is not possible to differentiate with certainty between actual synthesis by a tissue and its capacity to remove formed phospholipid from the plasma. Moreover, it is difficult to assess the synthetic capacities of tissues with low activities, such as muscle and brain, for it is possible that they derive appreciable amounts of phospholipid from tissues that show high activities.

The present investigation shows that phospholipid synthesis can be detected in surviving liver slices. This was made possible by the use of radioactive phosphorus as a labeling device; the sensitivity of the radioactive procedure for measuring phospholipid is such as to permit the accurate determination of traces of newly formed phospholipid. By this means it is now feasible to investigate the phospholipid metabolism of each tissue independently.

## EXPERIMENTAL

Male rats weighing between 180 and 220 gm. were sacrificed by a blow on the back of the neck and their livers rapidly removed.

\* Aided by a grant from the Research Board of the University of California.

Slices were prepared from the liver, and about 300 mg. of these were then carefully selected for uniformity and thickness, blotted on filter paper, weighed quickly, and transferred to a 50 cc. Erlenmeyer flask containing 5 cc. of a bicarbonate-Ringer's solution. The flasks were then placed in a constant temperature bath maintained at 37°.

The bicarbonate-Ringer's solution was prepared according to Krebs and Henseleit's method (5).<sup>1</sup>  $\text{KH}_2\text{PO}_4$  provided the only source of phosphorus in the solution. Enough radioactive phosphorus was used to provide 0.25 to 0.5 microcurie of radioactivity<sup>2</sup> per 5 cc. of solution. Each 5 cc. of solution contained 0.80 mg. of  $\text{KH}_2\text{PO}_4$  or 0.18 mg. of phosphorus. The bicarbonate-Ringer's solution was saturated with a gas mixture consisting of 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{O}_2$ , and the atmosphere above the solution was displaced with this gas mixture immediately after the addition of the slices to the flask. The pH of the solution was adjusted to 7.4 to 7.5 just before the addition of the slices. In a series of runs it was found that the pH at the end of 4 hours did not fall below 7.2.

*Extraction of Phospholipid from Tissue Slices*—Since the newly synthesized phospholipid was measured by its radioactivity, it was necessary to effect a complete separation between phospholipid and other phosphorus compounds. In these experiments the ratio of inorganic  $\text{P}^{32}$  to phospholipid  $\text{P}^{32}$  is obviously far greater than that encountered in studies involving the liver of the intact animal. Special precautions were therefore necessary to avoid contamination of radiophospholipid with inorganic radiophosphorus.

The contents of the flask, consisting of 5 cc. of bicarbonate-Ringer's solution and about 300 mg. of liver slices, were transferred quantitatively to a vessel containing 150 cc. of 3:1 alcohol-ether mixture. A flocculent precipitate appeared at this point, which consisted of tissue protein; and since it was highly radioactive, it also contained a large portion of the inorganic phosphate and probably other salts of the bicarbonate-Ringer's solution. The entire mixture was placed in a hot water bath maintained at 55° for

<sup>1</sup> In an earlier study (6) use was made of a phosphate-Ringer's solution. Since the incorporation of labeled phosphate into phospholipid was being measured, it was found desirable to employ a medium containing very small amounts of phosphate.

<sup>2</sup> 1 microcurie =  $4 \times 10^5$  arbitrary radioactive units, as measured with the Geiger counter.

2 hours and then filtered. The residue was reextracted with 100 cc. of the alcohol-ether solution for 1 hour and the mixture filtered. The combined extracts were concentrated under reduced pressure at 55°. The last stages of the concentration were allowed to proceed in an atmosphere of CO<sub>2</sub> until a volume of about 1 cc. was reached. At this point salts settle out; apparently a further separation of inorganic phosphorus occurs during concentration of the alcohol-ether extracts.

Ethyl ether was now added to the small residue in the concentration flasks, and the whole was thoroughly shaken and its contents quantitatively transferred to a glass-stoppered Erlenmeyer flask with a small side arm. The Erlenmeyer flask was rested on its side in a suitably constructed wooden rack to allow the aqueous layer to settle to the bottom. The supernatant ether layer was then poured off, while the heavier aqueous layer was caught in the side arm. An Erlenmeyer flask with a side arm used in this manner serves as a convenient separating device and is less cumbersome than the separatory funnel. The aqueous phase was extracted twice more in this manner by shaking with ethyl ether. The final aqueous residue was radioactive and contained most of the remaining inorganic phosphate.

It was frequently observed that ether extracts obtained as above were not clear. It seemed possible that small droplets of water were suspended in the ether and that this water contained appreciable amounts of inorganic phosphate. In order to remove this turbidity, the extracts were concentrated *in vacuo* at room temperature to a volume of about 10 or 15 cc. During this concentration a second aqueous phase usually appeared, leaving the ether solution above it quite clear. The concentration was carried out in an Erlenmeyer flask with a side arm, in order to facilitate the separation of the two layers. The aqueous residue was washed several times with ethyl ether. The ether extracts were then combined. They were quite clear and practically free of contaminating inorganic phosphate.

One further precaution was adopted to insure that none of the radioactivity of the ether layer was due to inorganic phosphate. The combined ether extracts were evaporated *in vacuo* at room temperature to a volume of about 15 or 20 cc., and about 100 mg. of finely powdered non-radioactive Na<sub>2</sub>HPO<sub>4</sub> added. The mixture was violently agitated for 5 to 10 minutes. After the solid

material had settled, the ether solution was decanted into another flask. The  $\text{Na}_2\text{HPO}_4$  residue was shaken two or three times with fresh portions of ether; these were decanted as before and added to the original ether extract. This new ether solution was now concentrated to a volume of about 15 to 20 cc., a fresh portion of inert  $\text{Na}_2\text{HPO}_4$  added, and the shaking and extracting repeated as above. The washing of the ether with inert  $\text{Na}_2\text{HPO}_4$  was repeated until the salt obtained after removal of the ether showed negligible radioactivity. A similar treatment with a saturated solution of  $\text{Na}_2\text{HPO}_4$  was finally carried out. The phospholipids in the ether solution were isolated and their radioactivity determined in the manner previously described (1).

The separation of radioactive inorganic phosphate from phospholipid was thus effected by the following steps: (1) diluting the reaction mixture with a large volume of an alcohol-ether mixture, whereby a considerable portion of the polar material precipitates out; (2) concentrating the alcohol-ether extract, a procedure that brings about a further precipitation of salts; (3) extracting the phospholipid from the residue with ethyl ether in which inorganic phosphate is not soluble even in the presence of phospholipid; (4) clarifying the ether solution by evaporating it to a small volume (at this point a water phase generally appeared, which permitted a further separation of inorganic phosphate); (5) removing the last traces of inorganic phosphate by several washings with inert  $\text{Na}_2\text{HPO}_4$ . This last procedure allows for an exchange of any radioactive inorganic phosphate present in the ether solution with the relatively huge amounts of added inert inorganic phosphate.

As a check on the separation of inorganic phosphate from phospholipid, the synthesis of radiophospholipid was examined at zero time as follows: liver slices were added to a bicarbonate-Ringer's solution containing inorganic phosphate. Immediately thereafter the entire mixture was poured into an alcohol-ether solution and phospholipids isolated in the manner described above. A negligible quantity of the radioactivity was recovered in the isolated phospholipids.

### Results

*Synthesis of Labeled Phospholipid by Liver Slices*—The formation of radiophospholipid by excised liver slices is shown in Table I. Labeled phospholipid was measured at 1, 2, and 4 hours. In

Columns 3 and 4 are shown the percentages of the initial radioactivity recovered as phospholipid per gm. of wet and dry tissue,<sup>3</sup>

TABLE I

*Synthesis of Labeled Phospholipid in Isolated Liver Slices of Normal Rats*

Rat No. (1)	Time interval (2)	Added P <sup>32</sup> recovered as phospholipid		Inorganic P of bicarbonate-Ringer's solution converted to phospholipid $\times 10^{10}$ <sup>a</sup>	
		Per gm. wet tissue (3)	Per gm. dried tissue (4)	Per gm. wet tissue (5)	Per gm. dried tissue (6)
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
L-1	1	0.68	2.9	1.2	5.2
L-2	1	0.75	3.2	1.4	5.8
L-3	1	0.71	3.0	1.3	5.4
L-109	1	0.88	3.7	1.6	6.7
L-110	1	1.3	5.5	2.3	9.9
L-111	1	1.2	5.0	2.2	9.0
L-112	1	1.2	5.0	2.2	9.0
L-105	2	3.2	13	5.8	23
L-106	2	3.5	15	6.3	27
L-107	2	2.6	11	4.7	20
L-108	2	4.5	19	8.1	34
L-4	2	2.2	9.2	4.0	17
L-5	2	2.4	10	4.3	18
L-6	2	2.8	12	5.0	22
L-7	2	2.6	11	4.7	20
L-8	2	2.9	12	5.2	22
L-9	2	2.5	11	4.5	20
L-10	4	5.2	22	9.4	40
L-11	4	4.9	21	8.8	38
L-12	4	4.3	18	7.8	32
L-101	4	5.4	23	9.7	41
L-102	4	8.9	37	16.0	67
L-103	4	5.7	24	10.0	43
L-104	4	5.2	22	9.4	40

\* Each bath had approximately 300 mg. of liver slices in 5 cc. of bicarbonate-Ringer's solution containing 0.18 mg. of P.

respectively. In Columns 5 and 6 is calculated the actual number of mg. of the added phosphorus in the bicarbonate-Ringer's solu-

<sup>3</sup> It should be noted here that the amount of liver slices as well as the amount of added phosphorus was kept constant in each vessel throughout this investigation. If this were not so, direct comparisons could not be made.



tion that had been converted to phospholipid. This does not represent the total amount of phosphorus converted to phospholipid, since the inorganic phosphate as well as other phosphorus precursors of phospholipid in the liver slices has been disregarded.

TABLE II

*Comparison of Phospholipid Synthesis by Liver Slices and Homogenized Liver*

Comparisons shown in the horizontal rows were made on the same liver.

Rat No.	Time in bath	Added P <sup>32</sup> recovered as phospholipid per gm. wet weight	
		Liver slices	Homogenized liver
	hrs.	per cent	per cent
1*	4	4.9	0.0
2*	4	6.0	0.0
3*	4	5.8	0.1
4	4	3.6	0.0
5	4	6.0	0.0
6	4	3.7	0.0
7	4	5.7	0.1
8	4	4.8	0.3
9	4	4.5	0.1
10	4	4.8	0.1
15	2		0.0
16	2		0.0
17	2		0.0
18	2		0.0
11	1		0.0
12	1		0.0
13	1		0.0
14	1		0.0

\* Liver ground by hand in an agate mortar. All others were homogenized in stainless steel apparatus.

*Synthesis of Labeled Phospholipid by Homogenized Liver*—In order to determine the effects of cell destruction, homogenized liver was prepared after the manner of Potter and Elvehjem (7). A stainless steel apparatus similar to that described by Colowick, Welch, and Cori (8) was employed. 1 gm. of rat liver was homogenized with 4 cc. of non-radioactive bicarbonate-Ringer's solution. 1 cc. of this suspension was added to 4 cc. of the radioactive bicarbonate-Ringer's solution prepared as above. In several cases, slices and

homogenized preparations were made from the *same* liver, and the phospholipid synthesis effected by them was determined simultaneously. Histological examination of homogenized preparations showed destruction of about 80 to 90 per cent of the liver cells. Table II shows the complete inability of destroyed hepatic cells to effect phospholipid synthesis. In order to rule out possible effects of metal, several samples of homogenized liver were prepared by grinding in an agate mortar, but these too failed to show phospholipid formation.

*Decomposition of Phospholipid in Isolated Liver Slices*—Two measures of phospholipid breakdown were examined in liver slices: (1) decreases in total phospholipid as determined with micro-

TABLE III  
*Breakdown of Phospholipids in Liver Slices*

Rat No.	Method of following phospholipid decomposition	Per cent of value at 0 hr. (100 per cent)		
		1 hr.	3 hrs.	6 hrs.
4	Labeled phospholipid	98	81	66
8-A	“ “	97	80	72
8-B	“ “	97	88	77
7	“ “	101	86	77
7	Total phospholipid	104	76	68
9	Labeled phospholipid	101	83	79
9	Total phospholipid	100	77	81

oxidative procedures (9), and (2) decreases of recently synthesized phospholipid that had been labeled with radiophosphorus. The latter measures the loss of radiophosphorus from the phospholipid molecule.

Rats were sacrificed 6 hours after the intraperitoneal injection of radioactive phosphorus. At this time considerable labeled phospholipid is deposited in the liver. About 350 mg. of slices were placed in flasks containing 5 cc. of Ringer's solution and treated as described above. The breakdown of phospholipid, both total and that recently synthesized from the injection of radiophosphorus, was then determined in each flask at intervals of 0, 1, 3, and 6 hours.

Practically no loss of phospholipid was observed at the 1st hour

(Table III). This was the case both for total and radiophospholipid. At the end of 6 hours about 30 per cent of phospholipid had decomposed.

#### DISCUSSION

In tracer studies with radioactive phosphorus, the entrance of phosphate into phospholipid is measured. Hence the term "synthesis" or "formation" as used in the present sense refers only to the over-all reaction in which inorganic phosphorus is converted to phospholipid regardless of the intermediate steps involved. According to this view, a new phospholipid molecule is formed by the addition of one or more of its five primary components to an appropriate residue. It is inconceivable that all five components unite simultaneously to form a new molecule. The various components may or may not be assembled in an orderly sequence such that the formation of one bond always precedes that of another. If the molecule is assembled in such a way that the formation of one bond always precedes that of another, then the rate at which phosphate is converted to phospholipid is also an index of the rate at which the other components enter into the molecule. If, however, a separate equilibrium is established for the formation and hydrolysis of each bond, it is very unlikely that the reaction rates for phosphate, fatty acid, and nitrogen will be the same. In the latter case it is to be expected that the rate of turnover for phospholipid phosphate differs from that of phospholipid fatty acid and that of phospholipid nitrogen. Furthermore, the relation of each of these to the other two may not be the same in different tissues.

Synthesis of phospholipid in liver slices was observed as early as 1 hour and continued for at least 4 hours. But no breakdown was observed in liver slices as early as 1 hour. Losses of 20 and 30 per cent, both radioactive and total, did occur in liver slices that survived for 3 and 6 hours, respectively. The fact that under these conditions the amount of labeled phospholipid showed no decrease for at least 1 hour points to the conclusion that no rapid enzymatic interchange between inorganic phosphate and phospholipid phosphate occurs.

It cannot be inferred, *a priori*, that the loss of radioactive P from the phospholipid molecule represents the same process as the loss

of oxidizable material in the phospholipid fraction, for non-radioactive phosphate from the medium might interchange with radio-phosphate of the phospholipid without affecting the molecule as a whole. In other words, the loss of labeled phosphorus may be involved in a reversible process, whereas the loss of total phospholipid may represent the complete disruption of the molecule. Both types of breakdown, however, involve the loss of phosphate. If only an interchange of phosphate took place, there would be a loss of phospholipid  $P^{32}$  but no loss of total phospholipid. The finding that both phospholipid  $P^{32}$  and total phospholipid disappear at rates that are roughly similar suggests that the reaction involving the complete disruption of the phospholipid molecule predominates.

The samples of radiophosphorus used in this investigation were prepared in the Berkeley cyclotron by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due. The assistance of Dr. C. Entenman in the determination of phospholipids by the oxidative procedure is gratefully acknowledged.

#### SUMMARY

1. The synthesis and destruction of phospholipid taking place concurrently in liver slices were studied in the present investigation. The former was measured by adding radioactive phosphate to the medium in which the liver slices were suspended. Breakdown was determined by adding, to a non-radioactive medium, liver slices obtained from rats that had received radiophosphorus approximately 6 hours before excision of their livers. Liver slices obtained from such animals contained radioactive phospholipid; the destruction of phospholipid was then measured by the decrease in their content of labeled phospholipid. The decomposition of phospholipid was also followed by the loss in *total* phospholipid.

2. The synthesis of phospholipid by surviving liver slices is demonstrated. Synthesis failed to occur in homogenized liver.

3. Synthesis was observed in the presence of a net decrease in the total phospholipid of the liver slice.

4. The significance of the conversion of inorganic phosphate to phospholipid phosphate is discussed.

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# ELECTROPHORETIC MEASUREMENTS ON NORMAL HUMAN PLASMA

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Comparative electrophoretic data on normal and pathological human plasmas have been published by a number of investigators (1-5). Normal human plasmas produce similar electrophoretic patterns, but the total number of cases published has not lent itself to establishing a normal range. If the electrophoretic method is to be of value in diagnosis or in measuring the alteration of the plasma picture, such as is observed in certain diseases, it is essential to ascertain the limits of variation in normal human plasma patterns. This paper represents a study of the electrophoretic patterns of twelve additional normal human plasmas and statistical treatment of a total of twenty-five directed toward the establishment of criteria for evaluating human plasmas. The mobilities of the plasma components were considered outside the scope of this discussion, although the values obtained on the twelve plasmas are included. Also, a heretofore unmentioned small component running ahead of the albumin is described.

## EXPERIMENTAL

The samples of plasma from non-fasting, normal laboratory workers of both sexes between 20 and 40 years of age were compared under equivalent conditions by the electrophoretic procedure. They were diluted 1:4 with a buffer consisting of 0.025 M lithium diethyl barbiturate, 0.025 M diethylbarbituric acid, and 0.025 M lithium chloride, at pH approximately 7.8, and dialyzed against 1 liter for 1 day and then 2 liters for a 2nd day. The electrophoretic patterns obtained by the scanning method of Longworth (6) were projected, magnified, and traced, and the

areas under the peaks were measured by means of a planimeter. This procedure was repeated twice in order to minimize the errors made in tracing and measuring the areas. It was found that the area measurements could be repeated to within about 4 per cent. The descending patterns of all components except the  $\beta$ -globulin were used. Inasmuch as the anomalous pattern of the descending  $\beta$ -boundary rendered measurements inaccurate, the ascending pattern was preferred.

Since with the buffer solutions used it is impossible to separate in a reasonable time the  $\gamma$ -globulin from the  $\delta$ - and  $\epsilon$ -boundaries of the buffer, the values given for the  $\gamma$ - include those for the  $\epsilon$ -boundary. This buffer boundary is known to be much less on the descending side than on the ascending side, because much of the protein remains immobile on the ascending side and produces an erroneously high  $\gamma$ -boundary (7). It is found, however, that the agreement between ascending and descending  $\alpha$ -globulin and fibrinogen areas is fair, indicating that only a small amount of the globulins remains at the  $\delta$ -boundary.

### Results

The results are collected in Table I and the standard deviations are figured for each set of values. Fig. 1 gives a graphic representation of the deviations for each of the components.

It is important to point out that the pattern for one plasma sample contains both a highest and a lowest component, and that no two of the highest components appear in the same pattern and likewise no two of the lowest components appear in the same pattern. This indicates that the variation of concentration introduced by dilution and dialysis was small, because such variations would cause uniformly high or uniformly low areas for all components of an individual pattern. It is not certain, however, that the area represented by a component is an accurate measurement of its nitrogen concentration, since the area depends on the product of the specific index of refraction of each component and its concentration.

In Table II are collected data of Longsworth *et al.* (1), Scudder (2), and the present series from which averages, standard deviations, and coefficients of variation are figured. From a statistical theory of random sampling it is possible to estimate the probable

limits within which various percentages of all normals should be found. Table III contains such estimates for three different percentages. From these data it should be understood that an analysis of a number of plasmas on any pathological condition should

TABLE I  
*Relative Concentrations and Mobilities of Components of Twelve Normal Plasmas*

Experiment No.	Areas, arbitrary units			Albumin*	$\alpha$ -Globulin	$\beta$ -Globulin (ascending)	Fibrinogen	$\gamma$ -Globulin	Mobilities $\times 10^5$ $\frac{\text{cm.}}{\text{sec.}}$ per volt/cm.				
	Total	Albumin	Globulin						Albumin	$\alpha$ -Globulin	$\beta$ -Globulin	Fibrinogen	$\gamma$ -Globulin
247	1387	954	433	2.44	0.092	0.181	0.045	0.135	6.4	5.3	3.3	2.4	0.7
249	1485	926	559	1.92	0.135	0.185	0.082	0.203	6.3	4.8	3.5	1.8	0.2
251	1211	748	463	1.87	0.100	0.234	0.084	0.201	6.4	5.0	3.4	1.8	0.0
253	1385	866	519	1.96	0.123	0.195	0.088	0.188	6.5	4.9	3.3	2.4	0.6
255	1344	810	534	1.92	0.142	0.211	0.102	0.204	7.1	5.4	2.9	1.9	0.5
342	1431	950	481	2.25	0.137	0.203	0.061	0.105	6.3	4.7	3.1	1.8	0.4
345	1403	908	495	2.09	0.146	0.194	0.066	0.139	6.7	4.9	3.3	2.4	0.9
349	1492	881	611	1.63	0.164	0.247	0.079	0.212	6.3	4.5	3.2	2.2	0.5
513	1421	829	592	1.52	0.143	0.300	0.069	0.202	6.4	4.7	2.7	1.5	0.2
514	1195	780	415	2.23	0.083	0.192	0.083	0.173	6.9	5.3	3.4	2.2	0.6
516	1341	825	516	1.91	0.115	0.219	0.103	0.116	7.2	5.3	3.5	2.1	0.4
519	1210	680	530	1.50	0.132	0.279	0.110	0.257	7.3	5.3	3.6	2.1	0.3
Average†	1359	847	512	1.94	0.126	0.220	0.081	0.178	6.6	5.0	3.3	2.1	0.4
S.D.‡	103	84.2	57.4	0.29	0.024	0.038	0.019	0.045	0.4	0.3	0.3	0.3	0.3
C. V., %§	7.6	10	11	15	19	17	23	25	6	6	9	14	(75)

\* Calculated on serum bases.

† Average = arithmetic mean =  $\Sigma x/N = \bar{x}$ .

‡ Standard deviation =  $\sqrt{\Sigma(x - \bar{x})^2/(N - 1)}$ .

§ Coefficient of variation = S.D./average.

be made before too much significance is placed on a pattern appearing abnormal but still falling within the estimated limits for normals.

In all of these normal plasma patterns there was observed a small component having a mobility of about  $18 \times 10^{-5}$  cm./sec. per volt/cm. which is here referred to as the  $\eta$  component. Unless



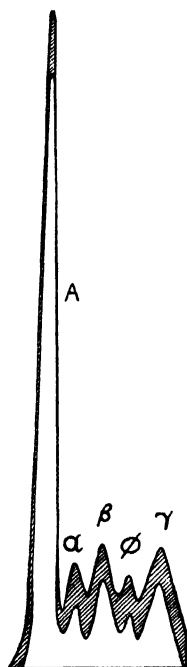


FIG. 1. The range of variation of components of twelve normal plasma patterns (shaded area). *A* = albumin;  $\alpha$  =  $\alpha$ -globulin;  $\beta$  =  $\beta$ -globulin;  $\gamma$  =  $\gamma$ -globulin;  $\phi$  = fibrinogen.

TABLE II

*Summary of Concentration Ratios from Twenty-Five Normal Plasma Patterns*

	Total No.	Albumin Globulin	$\alpha$ -Globulin Albumin	$\beta$ -Globulin Albumin	Fibrinogen* Albumin	$\gamma$ -Globulin Albumin
Longworth.....	7	1.99	0.125	0.23	0.09	0.21
Scudder.....	6	2.25	0.10	0.18	0.083	0.17
This series.....	12	1.94	0.126	0.22	0.081	0.19
Average.....	25	1.99	0.12	0.21	0.08	0.19
S.D.....		0.30	0.025	0.04	0.02	0.04
Coefficient of variation, %.....		15	21	19	25	21

\* Data available for only seventeen specimens.

a photograph was taken within 25 minutes after the voltage was applied, this boundary migrated out of view. Fig. 2, *a* illustrates this component in the plasma diluted 1:4 and Fig. 2, *b* shows the same plasma undiluted. The component appears in the undiluted plasma about 12 times as large as it does in that diluted

TABLE III

*Estimated Confidence Limits\* for 68, 94.5, and 99.7 Per Cent of All Normal Human Plasma*

Per cent	Albumin Globulin			$\alpha$ -Globulin Albumin			$\beta$ -Globulin Albumin			Fibrinogen Albumin			$\gamma$ -Globulin Albumin		
	68	94.5	99.7	68	94.5	99.7	68	94.5	99.7	68	94.5	99.7	68	94.5	99.7
Upper limit	2.39	2.79	3.46	0.150	0.190	0.240	0.260	0.320	0.410	0.110	0.140	0.170	0.250	0.300	0.39
Lower limit	1.59	1.10	0.520	0.090	0.050	0.000	0.160	0.100	0.010	0.050	0.020	0.000	0.130	0.080	0.00

\* Confidence limits are based upon estimations of standard errors of random samples. This theory does not imply that for the 99.7 per cent limits one must find three cases out of 1000 at the calculated limits, but that if there are more than three cases per 1000 outside these limits they are not normal.

$SE\bar{x}$  = standard error of average =  $SD/\sqrt{N-1}$

$SE_{SD}$  = " " " standard deviation =  $SD/\sqrt{2(N-1)}$

$\bar{x}$  = arithmetic mean

$\bar{x} + kSE\bar{x}$  = estimated upper limit of average for indicated percentage of all cases

$\bar{x} - kSE\bar{x}$  = estimated lower limit of average for indicated percentage of all cases

$SD + kSE_{SD}$  = estimated maximum of standard deviation

$\bar{x} + kSE\bar{x} + k(SD + kSE_{SD})$  = estimated upper limit of indicated percentage of all cases

$\bar{x} - kSE\bar{x} - k(SD + kSE_{SD})$  = estimated lower limit of indicated percentage of all cases

$k$  is given values of 1, 2, and 3 to calculate the limits for 68, 94.5, and 99.7 per cent, respectively, of all cases

1:4 and is much larger on the descending than on the ascending side. It is found in all human serum and plasma which have been investigated in this laboratory, but varies in amount from individual to individual. It is not observed in the presence of any other buffers (phosphate, borate, phosphate alkali) so far tried, except barbiturate; neither could it be observed with a 1:4 diluted

plasma when the barbiturate was increased to 0.1 M. An attempt was made to analyze this component by subjecting it to chemical tests. It gave a negative carbohydrate test but precipitated in both alcohol and acetone. Kjeldahl nitrogen determinations were not made because of the nitrogen introduced by the barbiturate buffer. Since the  $\eta$  component occurred only when this buffer was used, it is evidently a complex of some of the constituents of the serum and the buffer. If the barbiturate buffer is dialyzed

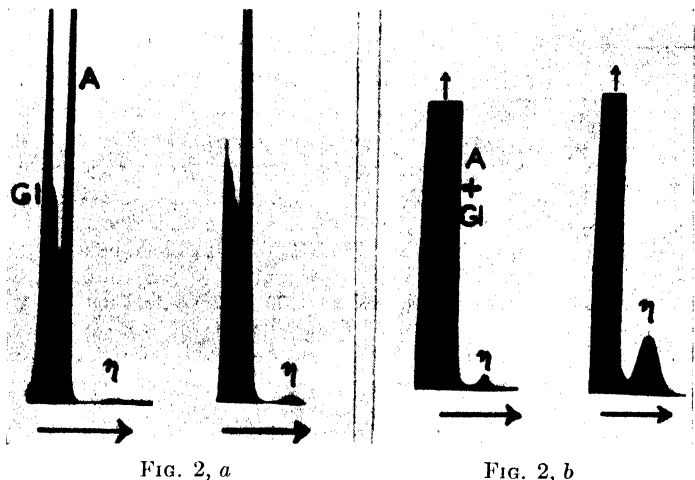


FIG. 2. Electrophoresis patterns showing fast component for normal plasma, after 8.4 volts per cm. were applied for 17 minutes. Left, ascending; right, descending. Fig. 2, *a*, diluted 1:4 with barbiturate buffer; Fig. 2, *b*, undiluted. *A* = albumin; *Gl* = globulin;  $\eta$  =  $\eta$  component.

away, the component apparently dissociates and is probably free and small enough to dialyze away also, because after the separated fraction was dialyzed against running water it could not again be detected. This along with the fact that its concentration increases so rapidly with increasing serum concentration is indicative of association-dissociation equilibrium. Also, the fact that it occurs so much more pronouncedly on the descending side, in the presence of serum, than on the ascending side, in the absence of serum, supports this explanation (8). Plasma was divided into water-soluble and water-insoluble fractions and in two experiments

the  $\eta$  component appeared only with the water-soluble fraction. The water-insoluble fraction, however, was analyzed only at low concentrations, because of the difficulty in redissolving it in the barbiturate buffer at pH 7.8.

We express our appreciation to Sheila Goldsmith, Gladys Gans, and Mary Wicks for their technical assistance, and our indebtedness to Dr. A. B. Gutman, Dr. Michael Heidelberger, and to Mr. Engle Devendorf for their interest and valuable criticisms.

#### SUMMARY

1. Electrophoretic data on twelve additional normal human plasmas are presented and a statistical treatment of the variations of electrophoretic components in twenty-five normal human plasmas is made.

2. Mention is made of a previously unobserved small component with high mobility usually occurring in plasma and serum in the presence of a barbiturate buffer.

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## THE ESTIMATION OF ADENINE

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Adenine has been determined gravimetrically as the picrate almost as long as the substance has been known. In 1890 Bruhns (1) described many of the properties of adenine and hypoxanthine, and worked out a procedure for their separation based ostensibly on the much greater solubility of hypoxanthine picrate as compared with adenine picrate. This method has been used extensively with good results (2, 3), although Wulff (4) soon found that the solubility of hypoxanthine picrate is very much less than Bruhns had supposed; the separation actually depends upon the fact that solutions of this substance can remain supersaturated for long periods.

According to Bruhns, a saturated solution of adenine picrate in water at 15–20° contains 5.2 mg. of adenine nitrogen per 100 cc. In the precipitation of this substance by the addition of a saturated solution of sodium picrate, the loss is inconsiderable because of the excess reagent. The loss incurred by washing the precipitate with water, however, becomes prohibitively large when the attempt is made to adapt the gravimetric method to the determination of small amounts of adenine. In working with 2 or 3 mg. of adenine nitrogen it has been the experience of workers in this laboratory that only about 90 per cent of the original material could be recovered. Tests with various organic liquids (*e.g.*, ethyl alcohol, acetone, ethyl acetate, 25 per cent acetic acid) have failed to disclose any washing medium that will remove the excess sodium picrate without dissolving a considerable amount of the precipitate.

The only substitute for the gravimetric determination of adenine as the picrate heretofore proposed is that of Parnas (5), in which

the picric acid is removed from the precipitate by extracting its hydrochloric acid solution with benzene, and the final analysis made by determining the total nitrogen in the residual aqueous layer. The accuracy of this method is problematical. Loss in manipulation is to a large extent avoided by washing (on the centrifuge) with only 1 cc. of water, hardly enough for a complete separation. No complete control analyses were presented by Parnas, and experiments in which both the picrate precipitate and the filtrate were analyzed (after benzene extraction) do not support the implied claim that the error amounts to only 2 per cent. In one sample of muscle, the picric acid precipitate was found to contain 7.02 mg. of purine nitrogen (aminopurine), the filtrate 17.6 mg. per cent (oxypurine). The sum is 24.63 mg. per cent, while the total purine nitrogen found by the Krüger-Schmid method on another aliquot of the same material was 27.65 mg. per cent, a discrepancy of 11 per cent.

An indirect micromethod for adenine involving a wholly different principle has been proposed by Schmidt (6). The combined amount of adenine and guanine is determined gasometrically, the guanine by means of guanase (7, 8), and the adenine by difference. The entire analysis takes several hours and requires special apparatus. The accuracy is estimated to be about 5 per cent with samples containing 0.5 mg. of purine nitrogen.

The procedure described in this paper, which permits the estimation of from 1 to 4 mg. of adenine nitrogen under suitable conditions with an error of less than 1 per cent, is volumetric, being based upon the direct acidimetric titration of adenine picrate (the salt of a strong acid and a weak base) in hot aqueous solution. It can be used for the analysis of solutions of adenine, of guanine (with somewhat less accuracy), or of mixtures of adenine and guanine (the result representing the sum of the two bases or adenine alone, depending on the circumstances), and for the separation of adenine from hypoxanthine (or xanthine). In the presence of more than a small amount of guanine, on the other hand, the separation from hypoxanthine cannot be effected by picrate precipitation, for two reasons. The precipitation of guanine by sodium picrate requires a higher concentration of the reagent and a longer time than is the case with adenine, and secondly the crystallization of hypoxanthine picrate can be induced

by seeding with guanine picrate (4). Both these circumstances are likely to result in partial precipitation of the hypoxanthine. The same restriction holds, of course, for the gravimetric method, or any other known procedure depending on the use of picrate.

The chief advantage of the titration method lies in the fact that the picrate precipitate can be washed with a sodium picrate solution instead of with water. The amount of substance lost in washing is thereby greatly diminished, while the sodium picrate left adhering to the washed precipitate does not interfere with the titration.

*Solutions Required—*

Sodium picrate solution for precipitation. A saturated solution of sodium picrate, the acidity of which is so adjusted (by adding picric acid solution) that, while it is distinctly acid to methyl red, not more than 0.2 cc. of 0.02 *N* sodium hydroxide is required in the titration of 10 cc. of the reagent to an orange color with phenol red.

Sodium picrate solution for washing. Dilute 50 cc. of the above solution to 100 cc.

0.02 *N* sodium hydroxide. Prepared free from carbonate and silicate, and kept in a paraffined bottle protected against atmospheric carbon dioxide (9).

Phenol red. A 0.05 per cent solution, adjusted if necessary so that 4 drops of the indicator in 10 cc. of water require no more than 0.01 cc. of 0.02 *N* acid or alkali to produce the full color change.

*Method*

The solution to be analyzed, containing 1 to 4 mg. of adenine nitrogen and not more than 5 mg. of adenine + hypoxanthine nitrogen, is placed in a large lipped Pyrex test-tube (200 × 25 mm.). If the starting material is the purine hydrochloride-hydrochloric acid solution obtained by decomposition of the copper precipitate (10), the excess hydrochloric acid is most easily removed before precipitation by the combined use of heat and a current of air. The Logan apparatus (11) is well suited to the purpose, but to avoid charring the heat must be turned off after the volume of solution has been reduced to about 5 cc. The dried residue is dissolved in 5 cc. of water. In some cases it may



be necessary, and is permissible, to add 1 or 2 drops of 0.5 N hydrochloric acid and warm to obtain a clear solution, cooling again to room temperature before proceeding further.

Add 1 cc. of the adjusted sodium picrate solution used for precipitation, and filter at once with suction through the small filtration tube described by Fiske (12) onto a mat of ashless filter paper pulp. Wash with two 1 cc. portions of the diluted sodium picrate solution. Transfer the filtration tube from the suction flask to the mouth of the test-tube in which the precipitation was carried out, introduce about 3 cc. of hot water into the filtration tube, stir up the precipitate and mat with a sharpened nichrome wire, and finally (with the same wire) poke the contents of the filtration tube through the small hole in the tip into the test-tube. Complete the transfer with the aid of a fine stream of hot water from a wash bottle, bringing the total volume of the solution to about 8 cc.

Add 2 drops of phenol red, heat the contents of the test-tube to boiling, and titrate at the boiling point with 0.02 N sodium hydroxide (added from a micro burette) to the first distinct red hue. 1 cc. of 0.02 N sodium hydroxide is equivalent to 1.40 mg. of adenine nitrogen.

### *Results*

Adenine is an amphoteric substance. When 8 mg. of adenine are dissolved in 10 cc. of boiling water, 0.02 cc. of 0.02 N sodium hydroxide is required to produce the first unmistakable red color, and about 0.08 cc. to bring out the full red color of the indicator. The titration of adenine picrate (or sulfate) to the first distinct red accordingly gives results which are slightly high (Experiments 1 to 4, Table I). The titration is substantially the same when the solution of an adenine salt is precipitated with sodium picrate and the washed precipitate titrated, according to the procedure given in the description of the method (Experiments 6 to 9). The average recovery throughout is 100.5 per cent. Indicators like brom-thymol blue and brom-cresol purple, which might otherwise be more satisfactory, cannot be used because the end-points are not sharply defined in the presence of picrate.

Table II shows the effectiveness of the separation of adenine and

hypoxanthine. In order to simulate the conditions encountered in the analysis of the copper precipitate (10) the mixtures of the two bases were evaporated to dryness after the addition of hydrochloric acid, and the picrate precipitation was carried out on an aqueous solution of the dried residue. The data given include a number of experiments showing the effect of varying the amount

TABLE I  
*Titration of Adenine Sulfate, Adenine Picrate, and Guanine Picrate*

Experiment No.	No. of determinations	Substance	Adenine N present	Recovery	
				Range	Average
			mg.	per cent	per cent
1	5	Adenine sulfate	3.015	99.9-100.8	100.3
2	1	" picrate	5.04		100.6
3	1	" "	6.23		100.8
4	1	" "	10.05		100.3
5	4	" " pptd.*	0.0501	100.7-103.5	102.2
6	4	Same as Experiment 5	0.997	100.4-101.4	100.7
7	4	" " " 5	2.00	100.1-100.8	100.5
8	5	" " " 5	3.015	99.8-100.8	100.2
9	4	" " " 5	4.00	100.1-100.8	100.6
10	4	Guanine picrate pptd.†	0.050	98.0-103.6	101.5
11	3	Same as Experiment 10	0.997	101.1-103.2	102.2
12	7	" " " 10	2.001	101.5-103.6	102.3
13	5	" " " 10	3.015	101.7-103.6	102.7

\* Precipitated from a total volume of 6 cc. containing 1 or 2 cc. of saturated sodium picrate solution. Filtration was begun within 30 seconds.

† Precipitated from a total volume of 6 cc. containing 3 cc. of saturated sodium picrate solution. The solutions were allowed to stand 30 minutes before filtration.

of sodium picrate solution added, as well as the time of precipitation. Under the conditions covered, either 1 or 2 cc. of saturated sodium picrate solution can be used with safety, while 3 cc. may precipitate some hypoxanthine when it is present in relatively large amounts, even if the filtration is begun at once. The risk of contamination with hypoxanthine when filtration is delayed is noticeably greater with 2 cc. than with 1 cc. of the reagent; hence the latter quantity is recommended for routine use.

*Guanine*

Guanine picrate is considerably more soluble than the corresponding salt of adenine (4, 13). This is shown clearly in Fig. 1, in which the solubilities of adenine and guanine picrates are shown as determined in the presence of various concentrations of excess picrate. In addition guanine picrate shares with hypoxanthine

TABLE II

*Separation of Adenine and Hypoxanthine*

All samples were precipitated from a total volume of 6 cc.

Purine N present		Saturated sodium picrate	Precipitation time	Adenine N found	Recovery
Adenine	Hypoxanthine				
mg.	mg.	cc.		mg.	per cent
0.997	2.001	1	30 min.	1.001	100.4
0.997	2.001	2	30 "	1.001	100.4
0.997	2.001	3	30 "	1.008	101.1
0.994	4.001	1	0	1.001	100.7
0.994	4.001	1	38 min.	0.994	100.0
0.994	4.001	1	19 hrs.	1.029	103.5
0.994	4.001	2	0	1.001	100.7
0.994	4.001	2	45 min.	1.008	101.4
0.994	4.001	2	18.5 hrs.	1.694	170.4
0.997	4.001	3	0	0.994	99.7
0.994	4.001	3	30 min.	1.015	102.1
0.994	4.001	3	18 hrs.	2.198	221.1
2.001	3.012	3	0	2.002	100.1
2.001	3.012	3	30 min.	2.114	105.7
2.001	5.011	1	0	2.009	100.4
2.001	5.011	2	0	2.009	100.4
2.001	5.011	2	16 min.	2.016	100.8
2.001	5.011	2	80 "	2.142	107.1
2.001	5.011	3	0	2.100	105.0

picrate the tendency to remain supersaturated, although to a much less marked degree. For these reasons the quantitative precipitation of guanine requires a higher concentration of sodium picrate than that recommended for the determination of adenine, and likewise more time. The results of Experiments 10 to 13, Table I, were obtained by using 3 cc. of saturated sodium picrate solution to 3 cc. of the purine base solution, the mixture being

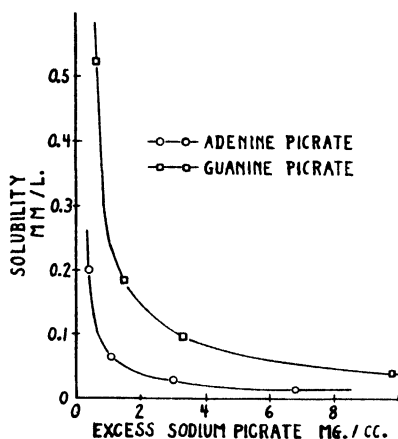


FIG. 1. The solubilities of adenine and guanine picrates in the presence of various concentrations of sodium picrate. The purine picrates were precipitated from about 30 cc. of dilute sodium picrate solution. After standing 24 hours at 23°, the mixtures were filtered and the amount of purine picrate precipitated was determined by titration of the precipitate.

TABLE III  
*Effect of Guanine*

5 cc. of the purine solution were precipitated with 1 cc. of saturated sodium picrate solution, and the filtration was started within approximately 30 seconds. Filtration and washing were completed within 3 to 5 minutes.

Purine N present			Adenine N found	Error
Adenine	Guanine	Hypoxanthine		
mg.	mg.	mg.	mg.	per cent
2.00	0.1	0	2.00	0
3.00	0.1	0	3.01*	+0.3
2.00	0.2	0	2.04	+2.0
2.00	0.5	0	2.17	+8.5
2.00	0.1	1.0	2.02	+1.0
3.00	0.1	1.0	3.01*	+0.3
2.00	0.2	1.0	2.11	+5.5

\* Precipitated with 2 cc. of saturated sodium picrate solution.

allowed to stand for 30 minutes before filtration was begun. The amount of alkali consumed in the titration is 2 or 3 per cent in excess of the calculated figure.

As stated earlier, the picrate method is excluded when guanine and hypoxanthine are simultaneously present unless the amount of guanine is quite small, as it would be in many protein-free tissue filtrates. In this case the method is useful only for the determination of adenine, under conditions designed as far as possible to prevent coprecipitation of the guanine. Any circumstance favoring the separation of guanine picrate, such as increasing the amount of picrate reagent above 1 cc. or allowing the mixture to stand at all before filtration, tends to contamination with hypoxanthine. As demonstrated in Table III, 2 or 3 mg. of adenine nitrogen can be separated from hypoxanthine in the presence of 0.1 mg. of guanine nitrogen. With twice as much guanine, which by itself under the conditions used would introduce little or no error, the simultaneous presence of hypoxanthine leads to definitely high results. Needless to say, this source of error is greatly magnified when the amount of guanine is still further increased.

#### *Preparation of Materials*

The adenine sulfate used, a product of the Eastman Kodak Company, was not subjected to further purification.

$(C_6H_5N_5)_2 \cdot H_2SO_4 \cdot 2H_2O$ .	Calculated.	N 34.65,	H <sub>2</sub> O 8.91
	Found.	" 34.68,	" 8.84

Adenine picrate was prepared from the sulfate by precipitation with sodium picrate. After being filtered and washed, it was recrystallized twice from 25 per cent acetic acid, and dried at 110°.

The preparations of adenine and of hypoxanthine have been described (14).

$C_6H_5N_5$ .	Calculated,	N 51.9;	found, N 52.0
$C_6H_4N_4O$ .	Calculated,	N 41.2;	found, N 41.3

Guanine hydrochloride was twice recrystallized from 2 N hydrochloric acid (see Hunter (15)) and dried at 105°.

$C_6H_5N_5O \cdot HCl$ .	Calculated,	N 37.3;	found, N 37.1
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#### SUMMARY

Adenine, and, under certain conditions, guanine, can be determined by precipitation with sodium picrate solution, and titration of the precipitates with standard sodium hydroxide solution.

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# A TITRIMETRIC METHOD FOR THE DETERMINATION OF SODIUM PREGNANEDIOL GLUCURONIDATE IN THE URINE OF PREGNANT WOMEN

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Studies of the metabolism of the sex hormones have been based for the most part on the determination of these compounds in the blood and urine. The estrogens and androgens are present in sufficient amounts so that they can be measured by bioassay but so little progesterone is present either in blood or urine that it is virtually impossible to detect it. A closely related compound, pregnanediol, is found in the urine but this is biologically inactive, so that its presence cannot be detected by bioassay. The compound must, therefore, be determined by some gravimetric, colorimetric, or other chemical procedure.

The first procedure devised and the one most widely used is that of Venning (1, 2). In this procedure the compound is isolated as the sodium salt of pregnanediol glucuronide and the amount present determined by weighing. A second method has also been devised by Astwood and Jones (3). In this procedure the urine is hydrolyzed by strong acid to deconjugate the sodium pregnanediol glucuronide and thereby liberate the pregnanediol. In this case pregnanediol rather than sodium pregnanediol glucuronide is determined by weighing. Both of these methods require the isolation of an essentially pure compound before the final gravimetric procedure can be employed.

\* The data in this paper were taken from the thesis submitted by E. Viergiver to the Graduate School of The University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



We have utilized still a third means of determining the pregnanediol. Since the compound is excreted as the glucuronide, it occurred to us that perhaps the compound could be hydrolyzed and the glucuronic acid so obtained be used as a measure of the pregnanediol excreted. Glucuronic acid has reducing properties similar to glucose and hence we thought that some one of the methods in use for the determination of glucose could be adapted for this purpose and, if so, that perhaps the sodium pregnanediol glucuronidate could be measured without isolation in the pure state. The method which is here described, based on these principles, is satisfactory and no more laborious than the ones devised by Venning and by Astwood and Jones and at least in our hands seems to be fully as accurate.

After several preliminary trials of various methods in common use for the determination of glucose we found the Shaffer-Hartmann-Somogyi (4) method the most satisfactory. The sodium pregnanediol glucuronidate was isolated from the urine essentially by the Venning method. The recovery of small amounts by precipitation from aqueous solution with acetone was rather low. So we sought some other means of precipitation. After some experimentation we found that the compound could be quantitatively precipitated from aqueous solution with lead nitrate. This compound could not be weighed as such nor could the lead content be used as a measure of the pregnanediol content because of the simultaneous precipitation of inorganic salts present, although with pure sodium pregnanediol glucuronidate the lead content was found to be an accurate indication of the amount of pregnanediol present. It was necessary, therefore, to recover the compound and measure the glucuronic acid. This was accomplished by decomposing the lead salt with sodium carbonate; a brownish solution was obtained in which the sodium pregnanediol glucuronidate could be readily determined by measurement of the reducing power before and after hydrolysis with acid.

#### *Method*

*Preparation of Urine Extracts*—A 24 hour specimen is collected under 300 cc. of butyl alcohol. The total volume, excluding the butyl alcohol, is noted and the urine is then extracted three times with volumes of butyl alcohol equal to one-third the volume of the

urine. The 300 cc. present during the collection are considered part of the first extraction and only enough butyl alcohol is added to make it equal to one-third the volume. After the solution is shaken, the layers are allowed to separate for 24 hours between extractions. The butyl alcohol extract is concentrated to a convenient volume, usually 500 cc., of which an aliquot sufficient to contain from 10 to 40 mg. of glucuronide is taken for analysis. In early pregnancy 300 cc. are usually required but after the 4th or 5th month 200 cc. are sufficient.

This aliquot of butyl alcohol is boiled to dryness *in vacuo* (water pump) and the residue is dissolved in 60 cc. of 0.1 N sodium hydroxide which is then extracted with three 30 cc. portions of butyl alcohol. This second butyl alcohol fraction is boiled to dryness, the residue is transferred to a 15 cc. centrifuge tube with approximately 10 cc. of distilled water, and 0.4 M lead nitrate is added until no further precipitation occurs. In routine practice it has been customary to add 1 cc. of the nitrate solution, which is more than sufficient in almost every case. After centrifuging, an additional drop or two is added to make certain that precipitation has been complete. The mother liquor is discarded and the precipitate is washed with 5 cc. of water containing a few drops of the lead nitrate solution. A glass rod is used to stir the precipitate and the same rod is used again in the subsequent extraction of the precipitate with sodium carbonate. The wash water is discarded and the precipitate is extracted with 0.08 N sodium carbonate made up in 60 per cent alcohol. The extractions are made first with 5 cc. of carbonate, and then twice with 2 cc. portions. The tube is warmed in hot tap water for 3 to 5 minutes and the precipitate is thoroughly broken up with the glass rod several times during the heating. The tube is centrifuged between extractions and the mother liquor is decanted into a 10 cc. volumetric flask. The final volume is made to 10 cc. with the alcoholic solution of sodium carbonate.

*Measurement of Amount of Glucuronic Acid*—To determine the amount of glucuronide a 2 cc. sample of the carbonate extract is transferred to a 10 cc. Pyrex volumetric flask. 1 cc. of 3.5 N hydrochloric acid is added, and the flask is stoppered with a small marble and heated 30 minutes in a boiling water bath. After cooling, the solution is neutralized with 1 N sodium hydroxide

with phenolphthalein as indicator. The volume is made to 10 cc. with water; the solution is shaken, and is then filtered without washing. Duplicate 4 cc. aliquots are placed in Pyrex test-tubes,  $25 \times 150$  mm. The blank is prepared by neutralizing 1 cc. of the acid, adding 2 cc. of the carbonate extract, and making the volume to 10 cc. Without filtering, duplicate 4 cc. aliquots are put in test-tubes.

2 cc. of Shaffer-Hartmann-Somogyi (4) reagent are added to all four tubes, after which they are stoppered with marbles and heated in a boiling water bath for 15 minutes. After the tubes have cooled a few minutes, 3 cc. of 1 N sulfuric acid are added, and the tubes are thoroughly shaken and titrated with 0.005 N sodium thiosulfate. The results are obtained by subtracting the number of cc. of 0.005 N thiosulfate used by the hydrolyzed tubes from the number of cc. used by the blanks; this figure is designated as  $(B - A)$ . The amount of sodium pregnanediol glucuronidate per cc. of stock solution taken for analysis, corresponding to various values of  $(B - A)$ , is obtained by inspection of Table I.

#### EXPERIMENTAL

*Establishment of Reference Curve (Table I)*—A standard reference curve was determined by measuring the amount of 0.005 N thiosulfate equivalent to varying amounts of pure sodium pregnanediol glucuronidate. Known amounts of the standard preparation were dissolved in 0.08 N sodium carbonate made up in 60 per cent alcohol and 2 cc. samples were hydrolyzed with 3.5 N hydrochloric acid. The solution was neutralized, made to 10 cc., and filtered. Duplicate 4 cc. aliquots were taken for the addition of the copper reagent as described above. The data when plotted graphically fell along a straight line (Fig. 1), the formula for which is  $y = 0.68x + 0.14$  as determined by the method of least squares. The amount of sodium pregnanediol glucuronidate per 1 cc. of stock solution originally taken for analysis is used rather than the amount actually present in the 4 cc. aliquot after hydrolysis, so that the results can be read directly without further calculation. The amount of material actually measured in each 4 cc. aliquot is eight-tenths of the amount present in 1 cc. of the original stock solution. With the formula given above, Table I was calculated for use in the same way that glucose values are obtained from similar tables.

*Optimum Conditions of Hydrolysis*—A series of experiments was carried out to determine the strength of acid and the length of

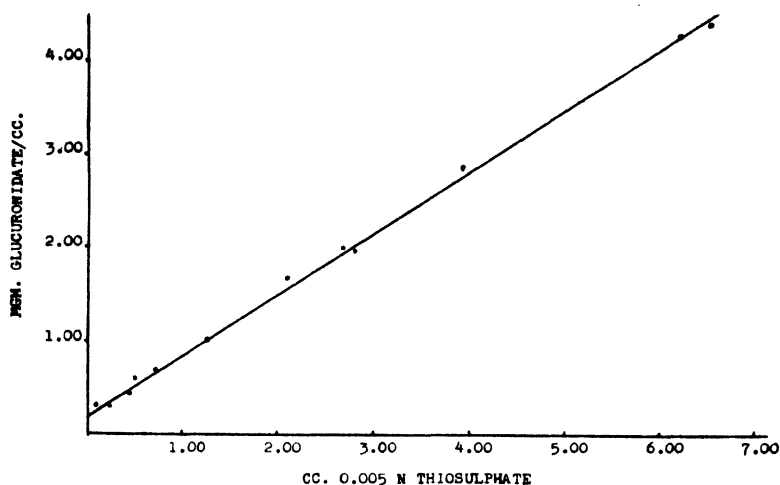


FIG. 1. The determination of the amount of 0.005 N sodium thiosulfate equivalent to varying amounts of sodium pregnanediol glucuronide.

TABLE I

*Amounts of Sodium Pregnanediol Glucuronide (Mg. in 1 Cc. of Sodium Carbonate Extract) Corresponding to Titration Values (B - A)*

0.005 N thiosul- fate (B - A)	Tenths of 1 cc. of 0.005 N thiosulfate									
	0	1	2	3	4	5	6	7	8	9
	Glucuronide per cc. stock solution									
cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0		0.21	0.28	0.34	0.41	0.48	0.55	0.62	0.68	0.75
1	0.82	0.89	0.96	1.02	1.09	1.16	1.23	1.30	1.36	1.43
2	1.50	1.57	1.64	1.70	1.77	1.84	1.91	1.98	2.04	2.11
3	2.18	2.25	2.32	2.38	2.45	2.52	2.59	2.66	2.72	2.79
4	2.86	2.93	3.00	3.06	3.13	3.20	3.27	3.34	3.40	3.47
5	3.54	3.61	3.68	3.74	3.81	3.88	3.95	4.02	4.08	4.15
6	4.22	4.29	4.36	4.42	4.49	4.56	4.63	4.70	4.76	4.83
7	4.90	4.97	5.04	5.10	5.17	5.24				

time required to give the best hydrolysis. The method in use at this time for measuring the glucuronic acid varied slightly from the final one, but the general conclusions can be applied equally

well to the latter procedure. Aqueous hydrochloric acid solutions were made up in varying strengths of known normality. 2 cc. samples of standard glucuronidate solutions containing approximately 1 mg. per cc. in alcoholic sodium carbonate were hydrolyzed with 1 cc. of acid for periods of 15, 20, and 30 minutes, and the equivalent amounts of thiosulfate were determined.

It was found that 15 minutes was not long enough for complete hydrolysis. 20 minutes gave results only slightly lower than for 30 minutes but it seemed advisable to use the latter for routine work, as the possibility of error is somewhat less. Hydrolysis for 1 hour showed no significant improvement over the 30 minute period. 2 cc. samples containing from 0.69 to 4.99 mg. per cc. of sodium pregnanediol glucuronidate were hydrolyzed for 30 minutes with 1 cc. of hydrochloric acid varying in strength from 0.5 to 7.0 N and the equivalent amounts of thiosulfate were determined. These results are shown in Table II. It is seen that the normality of acid giving the best results over the whole range of concentration of glucuronidate is either 3.0 or 3.5 N, and the latter was chosen for routine determinations. The stronger acids, 5 and 7 N, decreased markedly the amount of thiosulfate used. This is probably due to the presence of so much salt from the neutralization of the acid with sodium hydroxide.

Neither increasing nor decreasing the concentration of the alcohol content caused any significant change in the amount of thiosulfate required.

*Precipitation with Lead Nitrate*—Varying amounts of standard sodium pregnanediol glucuronidate were dissolved with the aid of heat in 10 cc. of distilled water plus 0.4 cc. of 0.1 N sodium hydroxide in a centrifuge tube. The alkali was added because in the preparation of extracts from urine the butyl alcohol fraction obtained from extraction of 0.1 N sodium hydroxide contains a small amount of alkali. The amount of sodium hydroxide to be added was determined by extracting 60 cc. portions of 0.1 N sodium hydroxide three times with 30 cc. portions of butyl alcohol. The butyl alcohol was boiled to dryness and the residue was dissolved in distilled water. The amount of alkali carried into the butyl fraction was determined by titration with standard acid and found to be the equivalent of about 0.4 cc. of 0.1 N sodium hydroxide.

This amount of alkali was consequently added to make conditions similar to those present in the precipitation of sodium pregnanediol glucuronidate with lead nitrate from extracts of urine.

The glucuronidate was precipitated with an excess of lead nitrate, the precipitate was extracted with alcoholic sodium carbonate, and the amount of glucuronidate recovered was measured by hydrolysis as described above. The final volume of carbonate used for the 5 mg. samples was 5 cc. instead of 10 cc. in order to increase the concentration of glucuronidate to approximately 1.0 mg. per cc. These results are shown in Table III. In one additional experiment not recorded in Table III the concentration

TABLE II

*Effect of Different Strengths of Acid on Hydrolysis of Sodium Pregnanediol Glucuronidate*

Amount hydrolyzed	Normality of acid during hydrolysis								
	0.16	0.33	0.60	0.83	1.00	1.16	1.33	1.66	2.33
	(B - A), cc. 0.005 N thiosulfate								
mg.									
0.69			0.24		0.18	0.27		0.16	
1.01	0.12	0.52	0.57	0.50	0.50	0.49		0.34	0.17
2.15		1.57	1.77		1.83			1.62	1.53
3.13		2.92	3.48		3.49			3.32	2.92
4.04			3.76	4.61	5.03	5.10	4.96	4.24	
4.99					6.30	6.27	5.82		

prior to precipitation was equivalent to only 1 mg. of glucuronidate per 10 cc. of water; yet the recovery was 73 per cent.

This study shows that the precipitation of the compound with lead nitrate under these conditions and the recovery from the lead salt by extraction with alcoholic sodium carbonate are virtually quantitative.

*Recovery from Alkali*—To determine the amount of butyl alcohol necessary to extract sodium pregnanediol glucuronidate from sodium hydroxide, samples of sodium pregnanediol glucuronidate weighing approximately 25 mg. were dissolved in 60 cc. portions of 0.1 N sodium hydroxide with the aid of heat and varying amounts of butyl alcohol were used to extract the compound. Three 30 cc. portions proved adequate for extracting the compound

and the butyl alcohol layer was large enough to be readily separated from the alkali.

To show that  $3 \times 30$  cc. of butyl alcohol are satisfactory, the recovery of varying amounts of glucuronidate was determined. Known amounts of the glucuronidate were dissolved in 60 cc.

TABLE III

*Recovery of Sodium Pregnanediol Glucuronidate by Precipitation with Lead Nitrate*

Experiment No.	Glucuronidate	Final volume of $\text{Na}_2\text{CO}_3$	Recovery	
	mg.	cc.	mg.	per cent
42	5.7	5	5.6	98.2
43	5.0	5	5.0	100.0
Average.....				99.1
41	10.1	10	9.0	89.1
			9.3	92.1
45	10.3	10	9.9	96.1
			9.8	95.1
Average.....				93.1
40	25.7	10	25.0	97.3
			25.2	98.1
44	25.0	10	24.6	98.4
			24.7	98.8
Average.....				98.2
58*	50.4	10	49.7	98.6
			47.6	92.2
Average.....				95.4

\* Precipitated from 24 cc. of water plus 1 cc. of 0.1 N sodium hydroxide.

portions of 0.1 N sodium hydroxide with the aid of heat and these alkaline fractions were extracted with  $3 \times 30$  cc. of butyl alcohol. After the solution was boiled to dryness, the residues from these butyl fractions were dissolved in distilled water and precipitated with lead nitrate. The precipitates were extracted with alcoholic sodium carbonate and the amount of glucuronic acid measured after hydrolysis. These results are shown in Table IV; the values

are absolute and are not corrected for any loss encountered in the precipitation with lead nitrate. When 5 to 10 mg. were added, the recovery varied from 76 to 98 per cent and when 25 to 50 mg. were added the recovery varied from 93 to 101 per cent. It

TABLE IV

*Recovery of Sodium Pregnanediol Glucuronide from 0.1 N Sodium Hydroxide*

Experiment No.	Glucuronide added	Recovery	
	mg.	mg.	per cent
51	5.0	4.9	98.0
54	5.3	4.4	83.0
57	5.0	3.9	78.0
Average .....			86.3
50	10.5	8.0	76.2
		8.0	76.2
53	9.9	8.7	87.9
		9.0	90.0
56	9.9	9.1	91.9
		8.2	82.8
Average .....			84.2
49	24.9	23.6	94.8
		24.2	97.2
52	25.8	24.7	95.7
		24.0	93.0
55	25.5	24.6	96.5
		25.8	101.2
Average .....			96.4
59	49.6	47.5	95.8
		46.5	93.8
Average .....			94.8

is apparent from these observations that even small amounts can be recovered from 0.1 N sodium hydroxide by this method without any great loss and with fair precision.

*Extraction of Urine Samples with Butyl Alcohol*—The volumes of butyl alcohol used by Venning for extraction of urine specimens are so small it is sometimes difficult to separate the two layers.



Extracting three times with portions of butyl alcohol equal to one-third the urine volume has proved satisfactory.

To determine whether this is sufficient to extract the conjugated compound completely, a fourth extraction was made which was analyzed separately from the first three. The amount of compound recovered in this fourth extract is negligible, as can be seen from the figures in Table V.

*Recovery from Urine*—The above studies indicated that each individual step in the method was satisfactory. It only remained to find out whether the compound, when added directly to unextracted urine, could be recovered. 24 hour specimens were

TABLE V

*Sodium Pregnanediol Glucuronidate Obtained in the Fourth Extraction with Butyl Alcohol*

Sample No.	Per 3 extractions	4th extraction	Per cent of total
	<i>mg.</i>	<i>mg.</i>	
A1	113.0	1.6	1.4
B2	100.3	3.4	3.4
A3	73.8	0.0	0.0
B4	48.8	0.0	0.0
A5	109.0	1.6	1.5
B6	87.5	1.7	2.0
A7	86.5	0.8	0.9
B8	120.5	2.7	2.2
C9	175.0	2.9	1.7

collected at home by clinic patients at varying periods of pregnancy. The urine was collected in the presence of only 75 cc. of butyl alcohol, because the specimens were to be divided into two equal fractions; a larger amount of butyl alcohol would form a separate layer, thus interfering with accurate sampling. The first extraction was made in the afternoon of the day upon which the collection ended, and it is evident from the figures obtained in the control fractions that no appreciable hydrolysis had occurred.

One-half of the specimen was used as a control and to the other half was added a known amount of sodium pregnanediol glucuronidate. The total amount of sodium pregnanediol glucuronidate in each fraction was then determined by the method outlined. The difference between the number of mg. found in the fraction

containing added glucuronidate and the number of mg. in the control fraction was considered the amount recovered.

The amount of standard glucuronidate added varied from 5 to 100 mg., and an attempt was made to measure the recovery of comparable amounts of compound from urines of both early and late pregnancy. Aliquots of butyl alcohol extracts were chosen to contain at least 10 mg., so that the final sodium carbonate solu-

TABLE VI  
*Recovery of Sodium Pregnanediol Glucuronidate from Urine*

Sample No.	Urine extracted	In Fraction A*	In Fraction B*	Added	Recovery	
	cc.	mg.	mg.	mg.	mg.	per cent
1 E.†	500	25.0	29.8	5.1	4.8	94.1
2 "	1000	26.8	32.0	4.9	5.2	106.1
4 L.	1000	42.2	46.0	5.4	4.3	79.6
5 E.	1000	46.3	54.2	10.0	7.9	79.0
6 L.	1100	100.3	113.0	10.1	12.7	125.7
7 "	890	131.8	140.3	10.5	8.5	81.0
8 E.	1000	29.0	52.9	25.0	23.9	95.6
9 L.	1140	48.8	73.8	25.6	25.0	97.6
10 "	1087	87.5	109.0	25.5	21.5	84.4
11 "	1000	103.5	127.0	25.3	23.5	92.9
12 E.	940	39.5	86.5	50.6	47.0	92.9
13 "	700	21.2	61.0	50.1	39.8	79.4
14 L.	1000	42.2	94.5	50.1	52.3	104.4
15 E.	1000	46.3	137.5	110.2	91.2	82.8
16 L.	800	82.8	174.5	100.4	91.7	91.3

\* Urine specimens were divided into two equal fractions. Fraction A was used as the control sample and known amounts of glucuronidate were added to Fraction B.

† E. designates specimens from early pregnancy, L. from late pregnancy.

tion would contain 1 mg., or more per cc. to increase the accuracy of the results. It has been the custom to concentrate the butyl extract of the urine to 500 cc. In order to obtain the concentrations mentioned, 300 cc. of this butyl alcohol concentrate are required when urine from early pregnancy is analyzed; only 100 cc. are required after the 4th or 5th month. If upon analysis less than 1 mg. per cc. was determined, the sample was analyzed again with a larger butyl alcohol aliquot. The results obtained by this procedure are shown in detail in Table VI. If the period

of gestation is disregarded, the average recoveries for the various amounts of glucuronidate added are as shown in Table VII. All figures are absolute and are not corrected for any loss that might occur in the extraction and precipitation. Inspection of Tables VI and VII shows that there is considerable variation in the per cent recovered but in no instance was the amount recovered less than 79 per cent of the amount added.

*Measurement of Interfering Substances*—Cohen and Marrian (5) have shown that estriol is excreted in the urine as the glucuronide. Androgens are also excreted in a conjugated form as shown by Peterson, Hoskins, Coffman, and Koch (6) and are known to be extracted from urine with butyl alcohol. If these conjugated compounds are extracted along with sodium preg-

TABLE VII  
*Average Recoveries of Glucuronidate from Urine*

Approximate glucuronidate added	Range of recovery	Average recovery
<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
5	80-106	93
10	79-126	95
25	84- 98	93
50	79-104	92
100	83- 91	87

nanediol glucuronidate, it is likely that they would also be precipitated by the lead nitrate and extracted by the sodium carbonate.

To show that the androgens and estriol are not found in any appreciable amounts in the sodium pregnanediol glucuronidate solution, the sodium carbonate extracts were analyzed for these compounds. Two butyl alcohol aliquots were chosen from samples of urine at various periods of gestation, and each was processed according to the procedure outlined under the method for preparation of urine extracts. The amount of sodium pregnanediol glucuronidate was measured in one of the carbonate solutions; the other, after removal of any free androgen and estriol by extraction with ether, was hydrolyzed and the androgens and estrogens separated by the method of Talbot and coworkers (7). The androgens were measured colorimetrically by the method of

Holtorff and Koch (8) and the estriol by a modification of the Kober procedure (9) in use in this laboratory. The recovery of standard dehydroisoandrosterone acetate by the above procedure was 78.0 per cent and that of standard estriol was 64.1 per cent.

The amounts of androgen (calculated as dehydroisoandrosterone) and of estriol are shown in Table VIII. It is evident that

TABLE VIII  
*Androgen and Estriol Content of Glucuronidate Solutions*

Sample No.	Duration of pregnancy	Amounts of hormones actually measured		Amounts of hormones corrected for loss	
		Androgen	Estriol	Androgen	Estriol
	<i>mos.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	1½	1.6	0.25	2.1	0.39
2	4	0.5	0.19	0.6	0.30
3	5	1.6	0.28	2.1	0.44
4	6	0.7	0.48	0.9	0.75
5	8	1.0	0.61	1.3	0.95
6	9	4.4	0.68	5.6	1.06

TABLE IX  
*Androgens Calculated As Glucuronidate*

Sample No.	Androgen as glucuronidate	Pregnanediol glucuronidate	Androgen * Pregnanediol
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	3.7	39.0	9.5
2	1.1	25.0	4.4
3	3.7	39.5	9.4
4	1.6	94.8	1.7
5	2.3	87.5	2.6
6	9.8	100.5	9.8

\* Mg. of androgen glucuronidate calculated as per cent of mg. of pregnanediol glucuronidate.

the amount of conjugated estriol in the sodium pregnanediol glucuronidate fraction is negligible.

If the androgen were conjugated with glucuronic acid as the sodium salt in the same way that pregnanediol is, the probable molecular weight would be 504. With this figure, the possible amounts of androgen as sodium dehydroisoandrosterone glucuronidate were calculated (Table IX). These results indicate that

the calculated amount of pregnanediol glucuronidate may be from 2 to 10 per cent too high owing to the presence of conjugated androgen in the solution.

### *Clinical Data*

The excretion of sodium pregnanediol glucuronidate in the urine in normal pregnancy has been determined by the method described above. The specimens were collected at home by the patient. Butyl alcohol was used as the preservative during the collection and specimens were extracted as soon as they were

TABLE X

*Excretion of Sodium Pregnanediol Glucuronidate in Urine of Pregnancy*

Duration of pregnancy	No. of determinations	Sodium pregnanediol glucuronidate		Calculated as pregnanediol, average per 24 hrs.
		Range per 24 hrs.	Average per 24 hrs.	
<i>mos.</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
3	1		44	26
4	5	50- 76	56	33
5	7	55- 80	68	41
6	5	76- 95	87	52
7	2	102-125	114	68
8	6	109-182	145	87
9	14	98-290	181	108

received, which was usually in the afternoon of the day on which the 24 hour period ended.

Urines from both early and late pregnancy have been analyzed and the results are shown in Table X. These values are the ones actually obtained and are not corrected for any losses. In order to compare these values with those of Venning, the mg. of sodium pregnanediol glucuronidate have been converted to mg. of pregnanediol, the factor 0.597, as determined by Venning (1), being used. It is evident that the order of magnitude of the results obtained with the lead precipitation method are within the range reported by those using the Venning method.

### DISCUSSION

The method herein described for the determination of sodium pregnanediol glucuronidate departs radically from the method

devised by Venning in so far as it utilizes the glucuronic acid present in the compound for the determination of the compound itself. The extraction from urine with butyl alcohol and partial purification by extraction from 0.1 N sodium hydroxide with butyl alcohol are essentially the same. From this point the procedure is different. The compound is recovered in the form of the lead salt from an aqueous solution by precipitation with lead nitrate, rather than as the sodium salt by precipitation with acetone. Precipitation with lead is virtually quantitative, even when the concentration is only 0.05 per cent. The sodium salt is recovered by digesting the lead salt with warm alcoholic sodium carbonate. The carbonate solution containing the sodium salt of pregnanediol glucuronide is then hydrolyzed under controlled conditions, thereby liberating the glucuronic acid which is determined by measuring its reducing capacity with Shaffer-Hartmann-Somogyi reagent. This procedure makes it possible to determine the compound without actually isolating it in relatively pure form as is necessary by the Venning procedure. The time required for carrying out the determination is about the same and the accuracy is probably also about the same.

The method has not been extensively used thus far for the determination of pregnanediol in normal or abnormal menstrual cycles. Enough observations, however, have been made to make it apparent that it may be of value. The main difficulty lies in the fact that the urine from non-pregnant women, even in the first half of the cycle when no sodium pregnanediol glucuronide is supposed to be present, contains a compound precipitable by lead which acts as a reducing agent only after hydrolysis with acid. A part of this is probably due to an androgen conjugated with glucuronic acid. The amount so measured, if calculated as sodium pregnanediol glucuronide, varies from 10 to 20 mg. per day.

The excretion of the compound in normal pregnancy, when measured by this procedure, was found to be about the same as reported by Venning. The average values obtained are almost identical, although the results here reported are not corrected for any losses entailed in the process, whereas the curve of excretion given by Venning is corrected for such losses.

The estriol, a considerable amount of which is present as the

glucuronide in the last third of pregnancy, is not determined by this method. The distribution between 0.1 N sodium hydroxide and butyl alcohol is such that the majority of the estriol glucuronide appears to remain in the alkali. If this were not so, it would doubtless be precipitated by the lead and would then be measured along with the pregnanediol because of its glucuronic acid content. A small but relatively constant amount of conjugated androgen is measured, however, as pregnanediol. This can be determined colorimetrically and deducted but the amount is so small, less than 10 per cent of the total, that it would probably have no significance in so far as interpretation of clinical results would be concerned.

#### SUMMARY

A new method for the determination of sodium pregnanediol glucuronidate in the urine of pregnant women is given. The method differs from other procedures in that the compound is precipitated as the lead salt from aqueous solutions and then finally determined by measuring the amount of glucuronic acid liberated by acid hydrolysis. The glucuronic acid is measured with the Shaffer-Hartmann-Somogyi alkaline copper reagent.

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# THE USE OF BACTERIA IN THE CHEMICAL DETERMINATION OF TOTAL VITAMIN C

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Based on the discovery of Esselen and Fuller (3) that certain strains of *Bacterium coli* are able to reduce dehydroascorbic acid, a simple rapid method has been worked out for the determination of vitamin C in food products. After 15 minutes incubation with a suspension of resting cells the total vitamin C in the form of ascorbic acid is titrated directly with 2,6-dichlorophenol indophenol dye.

The procedure, as applied to milk, is as follows: 10 cc. of milk are measured into a test-tube containing 0.5 to 1.0 cc. of *Bacterium coli* suspension. After mixing, the contents of the tube are brought to 40° and held 15 minutes. The tube is then emptied into a beaker and rinsed with 20 cc. of 0.1 N H<sub>2</sub>SO<sub>4</sub>. The acidified solution is titrated immediately with dye to an end-point remaining definitely pink for 30 seconds (7). Various acids may be used for acidification and the solution may be filtered before titration, although this additional step is usually unnecessary. For the determination of total vitamin C in food products other than milk, the extract or juice should be brought to pH 6.2 before reduction. The bacteria are more active if 1.0 cc. of cell suspension is mixed with 1.0 cc. of 10 per cent glucose 15 minutes before addition of the extract to be analyzed. Pretreatment of the bacterial suspension with glucose is not necessary when milk is the material for analysis.

The *Bacterium coli* suspension is prepared as follows: An 18 to 24 hour culture of *Bacterium coli* is added to 5 liters of broth containing 1.0 per cent peptone, 0.1 per cent glucose, and 0.3 per cent secondary potassium phosphate and allowed to grow



14 to 24 hours at 37°. The culture is centrifuged in a continuous centrifuge, the cells washed once with 0.03 M neutral phosphate, then suspended in 50 cc. of the neutral phosphate, and stored in a refrigerator. There is no appreciable deterioration in activity of the cell suspension in 1 month if it is kept cold.

Approximately one-half the strains of *Bacterium coli* isolated from human feces are active in reducing dehydroascorbic acid but the individual strains show considerable differences in activity. A strain designated as "Crookes," originally presented to the authors through the kindness of Dr. Esselen, has proved consistently active. Besides *Bacterium coli* certain strains of the closely related *Bacterium cloacae* were also found to reduce dehydroascorbic acid. Negative results were obtained with fifteen strains of streptococci and with bakers' yeast.

*Bacterium coli* is to a certain extent a specific biological reagent for the determination of oxidized vitamin C in that no oxidation product of ascorbic acid is reduced except dehydroascorbic acid. Oxidation of ascorbic acid with hydrogen peroxide, or treatment of dehydroascorbic acid with alkali or heat yields products which no longer can be reduced with these bacteria. However, the reducing action of *Bacterium coli* is not entirely specific for oxidized vitamin C, because the oxidation product of *d*-isoascorbic acid can also be reduced. See Table I.

The necessary conditions for the quantitative reduction of dehydroascorbic acid have been elaborated. The sensitivity of the acid to heat and alkali offers the chief difficulty. If conditions of pH and temperature for the most rapid bacterial action are selected, the dehydroascorbic acid disappears so quickly that complete reduction is not obtained.

The more alkaline the pH and the higher the temperature the more rapidly the reduction must be carried out to avoid loss. In milk at pH 6.6 and 40° quantitative reduction can be brought about in 10 to 15 minutes. In fruit juices adjustment to pH 6.2 makes possible complete reduction with minimal quantities of bacterial suspension. Sufficient cells must be used to insure rapid reduction. If the reduction is not 100 per cent complete within 15 minutes, it never will be complete. The amount of cell suspension needed in a determination should be tested each time the cells are grown and every few days during storage.

The activity of *Bacterium coli* suspensions is tested with a standard dehydroascorbic acid solution prepared just before use by adding sufficient 0.01 N iodine exactly to oxidize a few mg. of solid ascorbic acid (4). Since ascorbic acid and iodine react quantitatively, this solution can be used as a standard solution of 0.01 N dehydroascorbic acid. 1 cc. portions, containing 0.88 mg. of dehydroascorbic acid, are added to 10 cc. of buffer containing glucose and bacterial suspension. The reaction between ascorbic acid and iodine is a simple reversible oxidation. We have found in six determinations that 1.0 mole of ascorbic acid reacts with 0.997 mole of iodine,  $\pm 0.007$  mole maximum deviation.

TABLE I

*Reduction of Dehydroisoascorbic Acid and Dehydroascorbic Acid by Bacterium coli*

Temperature, 40°; the solutions contain 20 cc. of phosphate buffer, 0.05 M, pH 6.4, 4 cc. of 10 per cent glucose, 2 cc. of suspension of *Bacterium coli*, 4.3 mg. of dehydroascorbic acid in one solution, and 4.3 mg. of dehydroisoascorbic acid in the other.

Time	Ascorbic acid	Isoascorbic acid
min.	mg.	mg.
Start	0	0
5	0.42	0.14
10	0.84	0.24
20	1.89	0.70
30	2.45	0.91
70	2.98	1.50

The purity of the ascorbic acid was tested by alkaline titration. The average of five analyses was  $100.01 \pm 0.69$  per cent maximum deviation.

A large number of experiments have demonstrated that reduction of dehydroascorbic acid by *Bacterium coli* is 100 per cent complete in the pH range 6.2 to 6.6 at 40°. For example, with two different strains of *Bacterium coli* in a series of twenty determinations, an average of 99.5 per cent reversal ( $\pm 1.9$  per cent maximum deviation) was obtained. Complete reduction of dehydroascorbic acid by *Bacterium coli* was also obtained when the ascorbic acid had been oxidized by oxygen with the aid of ascorbic acid oxidase from cucumber juice. The oxidation of 0.01 N ascorbic acid with

1.0 per cent of cucumber juice concentrate was carried out at 10° in 0.05 M phthalate buffer of pH 5.0. As soon as the oxidation was complete, 1.0 cc. of the dehydroascorbic acid solution was added to 10 cc. of phosphate buffer of pH 6.2 containing bacterial suspension and glucose. Reduction was allowed to take place at 40°. In sixteen determinations the average per cent reduction was  $98.6 \pm 3.7$  maximum deviation. This figure is slightly lower than 100 per cent because of unavoidable loss of dehydroascorbic acid during the time required for oxidation.

TABLE II

*Comparison of Reducing Action of Bacterium coli and H<sub>2</sub>S after Oxidation of Ascorbic Acid by Non-Specific Reagents*

All solutions contained 0.88 mg. of ascorbic acid per cc. and solutions with H<sub>2</sub>O<sub>2</sub> contained 0.3 mg. of H<sub>2</sub>O<sub>2</sub> per cc.

Oxidizing agent	Ascorbic acid after partial oxidation	Ascorbic acid after reduction by <i>Bacterium</i> <i>coli</i> 15 min. at pH 6.2	Ascorbic acid after reduction by H <sub>2</sub> S 20 min. at pH 4.0	Ascorbic acid after reduction by H <sub>2</sub> S 24 hrs. at pH 4.0
	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.
H <sub>2</sub> O <sub>2</sub> , unbuffered.....	0.046	0.14	0.13	0.21
O <sub>2</sub> , ".....	0.61	0.69	0.69	0.73
H <sub>2</sub> O <sub>2</sub> , pH 5.0.....	0.008	0.054	0.054	0.096
O <sub>2</sub> , " 5.0.....	0.60	0.69	0.70	0.73
H <sub>2</sub> O <sub>2</sub> , " 7.0.....	0	0	0.004	0.009
O <sub>2</sub> , " 7.0.....	0.41	0.43	0.42	0.44
H <sub>2</sub> O <sub>2</sub> , " 10.0.....	0.079	0.082	0.086	0.086
O <sub>2</sub> , " 10.0.....	0.19	0.19	0.19	0.24

The method generally used at present for the chemical determination of total vitamin C is by means of reduction with hydrogen sulfide (1,2,5,8). Mack and Tressler (6) have observed a non-specific action of hydrogen sulfide on biological materials by which reducing agents capable of reacting with the dye are produced. We have compared the reduction by *Bacterium coli* with the reduction by H<sub>2</sub>S. If the reduction by H<sub>2</sub>S is carried out at pH 4.0 in pure buffered solutions (free of biological materials), the two methods give the same results (see Table II). This agreement between the two methods holds even when non-specific oxidizing agents such as copper or hydrogen peroxide cause the destruction of the ascorbic acid. We have no evidence to support the sug-

TABLE III

*Ascorbic Acid and Dehydroascorbic Acid in Commercial Milk*

The values are given in mg. per liter.

	Days after milking							
	1		2		4		6	
	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid
Raw.....	14.9	6.3	11.4	4.8	5.1	7.9	1.7	4.7
Pasteurized.....	14.9	3.6	10.4	4.8	3.8	7.9	2.4	2.6
"    0.1 mg.								
Cu per liter.....	7.9	8.6	2.1	8.9	0	5.1	0	1.0
Pasteurized, 0.5 mg.								
Cu per liter.....	0	12.6	0	7.0	0	3.4	0	1.0
Pasteurized, 1.0 mg.								
Cu per liter.....	0	9.6	0	4.9	0	3.1	0	0.7

TABLE IV

*Reduced and Total Vitamin C in Commercial Canned Juices*

The values are given in mg. per cc. of undiluted juice.

Juice	pH, undiluted juice	0.2 N NaOH to bring 1 cc. to pH 6.2	Immediately on opening can		After aeration and 5 days storage in glass at 1°			
			Ascorbic acid	Total vitamin C	Ascorbic acid	Total vitamin C	Total vitamin C after addition of 0.335 mg. dehydroascorbic acid	Recovery of added dehydroascorbic acid
		cc.						per cent
Orange.....	3.8	0.48	0.393	0.394	0.364	0.395	0.716	95.8
Grapefruit....	3.8	0.92	0.344	0.342	0.307	0.321	0.652	98.9
Lemon.....	2.5	4.35	0.330	0.330	0.170	0.302	0.635	99.4
Pineapple.....	3.6	0.53	0.084	0.092	0.075	0.084	0.419	100
Apple.....	3.5	0.46	0.004	0.006	0	0.016	0.349	99.4
Sauerkraut....	3.9	0.36	0.153	0.007	0.037	0	0	0
Composite vegetable...	4.4	0.22			0.013	0	0.031	9.3

gestion of Tillmans (8) and Woessner, Elvehjem, and Schuette (9) that non-specific oxidizing agents besides causing the irreversible destruction of ascorbic acid will also cause the formation

of substances other than dehydroascorbic acid which, when reduced with  $H_2S$ , are capable of instantaneous reaction with indophenol at pH 4.0.

Tables III and IV show application of the method of bacterial reduction to milk and canned fruit juices. In the case of commercial milk samples titration of the reduced ascorbic acid alone is not even an approximate measure of total vitamin C. The method of bacterial reduction is not applicable to all biological materials. For example, in sauerkraut and a composite vegetable juice the *Bacterium coli* failed to cause any reduction of the added dehydroascorbic acid but acted instead as an oxidation catalyst to decrease the ascorbic acid present. This reversal of the action of the bacteria in some biological fluids has not been explained. The bacterial reduction method for total vitamin C can be applied satisfactorily to urine. In a typical analysis 1 cc. of urine was found to contain 0.027 mg. of ascorbic acid and 0.053 mg. of total vitamin C. Added dehydroascorbic acid was recovered to the extent of 99.4 per cent.

#### SUMMARY

The quantitative determination of total vitamin C in biological materials can be accomplished by reduction of dehydroascorbic acid to ascorbic acid with a resting suspension of *Bacterium coli*, followed by direct titration of the ascorbic acid with 2,6-dichlorophenol indophenol in acid solution. The reduction can be carried out in 15 minutes at pH 6.2 to 6.6 and 40° with a suspension of the bacteria in the presence of glucose.

The method has been applied to milk, fruit juices, and urine.

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## THE ESTIMATION OF THIAMINE IN URINE

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In recent years many papers on methods for the estimation of thiamine (vitamin B<sub>1</sub>) in the urine have been published. The most important of these contributions have been those of Jansen (1), Melnick and Field (2), Hennessey and Cerecedo (3), and Schultz *et al.* (4). Each of these methods contributes definite advantages from the chemical point of view, although the last three do not lend themselves readily to clinical use. The present paper presents a method based on the oxidative conversion of thiamine to thiochrome with a critical study of the various steps in the procedure both in respect to the present modification and to those of the foregoing authors. Elsewhere a report on the use of the method in clinical investigation will be given.<sup>1</sup> Over 680 estimations of thiamine in urine have been performed with this technique and our experience leads us to believe that it is satisfactory for investigative purposes.

### *Procedure*

#### *Reagents—*

Glacial acetic acid (C.P. Baker's Analyzed).

Acetic acid 0.2 N solution and a 1 per cent solution.

Isobutyl alcohol (Merck) (must be free from fluorescence in ultraviolet light and frequently tested for this property).

Potassium chloride (Baker's Analyzed), a 25 per cent solution.

Potassium ferricyanide (C.P. Baker's Analyzed), a 0.5 per cent solution prepared fresh daily.

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<sup>1</sup> Egaña, E., unpublished data.

Sodium hydroxide (c.p. Baker's Analyzed), a 20 per cent solution.

"Decalso."<sup>2</sup>

Thiamine hydrochloride (Merck, crystals).<sup>3</sup>

*Method*—The urine is collected with glacial acetic acid as a preservative. 10 ml. of acetic acid are sufficient for each 24 hour amount. The resultant pH under these circumstances is approximately 5. Urines so preserved show no loss of thiamine in 48 days. 12 ml. of the acidified urine are pipetted into a special centrifuge tube<sup>4</sup> fitted with a ground glass stopper, and shaken with 10 ml. of isobutyl alcohol, to remove fluorescent interfering substances. When thiamine is being fed, or when unusually large amounts of the vitamin are excreted into the urine, 0.1 to 5 ml. samples are used and the initial washing with isobutyl alcohol omitted. This omission under such circumstances will not introduce appreciable errors, since the amount of thiamine present is high in relation to the fluorescent interfering substances. The tube and contents are centrifuged for 5 minutes to separate the two layers. The isobutyl alcohol has the property of absorbing a portion of the aqueous layer. The aqueous layer is therefore measured and a 9 ml. aliquot removed to a test-tube and 10 ml. of distilled water added. The pH of the resulting mixture is brought to 4.2 to 4.5 with 0.2 N acetic acid, brom-cresol green being used as an external indicator. Failure to adjust to this pH will result in incomplete adsorption.

The thiamine is then adsorbed from the diluted and acidified urine by Decalso. (The amount of Decalso is important; less than 140 mg. will result in incomplete adsorption, and more than 300 mg. requires the use of too much oxidant later in the procedure.) Approximately 200 mg. of Decalso are shaken into 10

<sup>2</sup> Obtained from The Permutit Company, New York, and ground through a standard 100 mesh sieve. It is then given two washings with 1 per cent acetic acid followed by two washings in 95 per cent ethyl alcohol and finally washed free from fluorescent substances with acetone. This material was first used by Hennessey and Cerecedo (5) but was not freed from fluorescent impurities.

<sup>3</sup> According to Merck and Company, the crystals of thiamine hydrochloride contain 4 per cent hygroscopic water.

<sup>4</sup> Obtainable from the Tamworth Associates, Needham Heights, Massachusetts.

ml. of 1 per cent acetic acid<sup>5</sup> and transferred to a Gooch crucible fitted with a 2 cm. filter paper. This amount of Decalso is sufficient for 0.1 to 30 ml. urine samples. A good source of vacuum must be provided. The suspension of Decalso is poured into the Gooch crucible and while 0.5 to 1 ml. of liquid still remains above the Decalso layer, the urine sample is added to the contents of the crucible. Adsorption is complete in about 1 minute. While 0.5 to 1 ml. of uriferous liquid remains above the Decalso, the adsorbate is washed by suction with 10 ml. of 1 per cent acetic acid. The Decalso together with the filter paper is then transferred to the special ground glass-stoppered centrifuge tubes. Traces of Decalso left on the walls of the crucible are washed into the centrifuge tube with 2 ml. of 25 per cent potassium chloride solution. The potassium chloride-Decalso mixture is shaken gently to complete elution. Depending on the amount of vitamin B<sub>1</sub> one expects to find, from 0.2 to 0.5 ml. of potassium ferricyanide solution is added, and finally 1 ml. of 20 per cent sodium hydroxide. The order of addition of reagents is important to prevent overoxidation. The resultant pH is approximately 10.6, which is the optimal condition for thiochrome production. The mixture is shaken for half a minute. If the change of color from yellow to white is extremely fast, indicating that an excess of thiamine is present, it is then necessary to add more potassium ferricyanide drop by drop until a slight yellow coloration remains, thus indicating that a sufficient amount of the oxidizing agent is present. 4.9 ml. of isobutyl alcohol are then added. (Due to the removal of water from the aqueous layer, the resultant volume of the isobutyl alcohol layer will be 5 ml.) The centrifuge tube is then shaken vigorously for 1 minute and the layers separated by centrifuging.

2 ml. of the isobutyl alcohol layer are pipetted into a Pyrex test-tube (10 × 0.8 cm.). The fluorescence exhibited by the contents of the tube in an ultraviolet source<sup>6</sup> is compared visually with identical but sealed tubes containing known amounts of synthetic thiochrome in 2 ml. of isobutyl alcohol as standards.

<sup>5</sup> Since Decalso can adsorb hydrogen ions, acetic acid suspensions must be used to prevent increase in alkalinity of the urine with consequent failure of complete adsorption.

<sup>6</sup> Mercury vapor ultraviolet lamp obtainable from the Central Scientific Company, Cambridge, Massachusetts.



*For reasons given below, when urines from individuals suspected of having vitamin B<sub>1</sub> deficiency or urines containing bile or blood are analyzed, a known amount of thiamine hydrochloride should be added to a duplicate sample of urine and carried through the procedure.*

*Preparation of Thiochrome Standards*—Theoretically 1  $\gamma$  of thiamine should yield 0.777  $\gamma$  of thiochrome, if all the thiamine were oxidized to thiochrome. It has been our experience that this conversion is not complete. An attempt was made, therefore, to determine whether the percentage of thiamine converted to thiochrome from aqueous solutions was constant for all amounts of thiamine within the range usually studied by the present analytical

TABLE I

*Conversion of Thiamine Hydrochloride to Thiochrome in Aqueous Solution*

Thiamine hydrochloride present	Thiamine present as thiochrome*	Thiochrome found	Thiamine converted to thiochrome
$\gamma$	$\gamma$	$\gamma$	per cent
0.4	0.31	0.19	61
0.5	0.38	0.24	63
0.6	0.46	0.29	63
0.8	0.62	0.39	63
1.0	0.77	0.49	64
1.2	0.92	0.59	64
1.5	1.11	0.74	66
1.8	1.39	0.89	64
2.0	1.54	0.99	64

\* 1  $\gamma$  of thiamine hydrochloride equals 0.77  $\gamma$  of thiochrome.

procedure. A consideration of the data presented in Table I indicates quite clearly that this is the case. The data show that only about 65 per cent of the thiamine is converted to thiochrome when oxidized by potassium ferricyanide. This yield is considerably higher than that obtained by other workers (6-10).

The thiochrome standards were prepared in the following manner: Approximately 1 mg. of thiochrome was weighed out on a micro balance and dissolved in an amount of isobutyl alcohol to give a solution having a final concentration of 10  $\gamma$  per ml. (Solution 1). By dilution from this solution a second solution was prepared in which the concentration of thiochrome is 1  $\gamma$  per ml. (Solution 2). From this solution by dilution a third solution

containing 0.1  $\gamma$  per ml. was prepared (Solution 3). By the use of appropriate amounts of these three solutions of thiochrome and appropriate amounts of isobutyl alcohol a final set of standards was prepared ranging from 0.1 to 2  $\gamma$  in 0.1  $\gamma$  intervals. The tubes containing the standards were then sealed. The amount of thiochrome used in each standard tube should be contained in a total volume of 5 ml. and each tube should contain exactly 2 ml. of this solution. Needless to say, the standard tubes and those used for the final observation of the unknown should be free from fluorescence and of the same dimensions.

TABLE II

*Recovery of Added Thiamine Hydrochloride from Normal Urine*

0.15  $\gamma$  of thiamine as thiochrome in original urine sample.

Thiamine added	Thiamine added expressed as thiochrome*	Total thiochrome found	Thiamine added recovered as thiochrome	Recovery†
$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
0.20	0.15	0.23	0.08	92
0.50	0.38	0.40	0.25	101
0.75	0.58	0.50	0.35	92
1.00	0.77	0.65	0.50	100
2.00	1.54	1.16	1.01	100

\* 1  $\gamma$  of thiamine hydrochloride equals 0.77  $\gamma$  of thiochrome.

† With a conversion factor of 65 per cent.

In labeling the tubes with their thiamine equivalent it must be borne in mind not only that the molecular equivalent of 1  $\gamma$  of thiamine is equal to 0.777  $\gamma$  of thiochrome but also the fact that there is only a 65 per cent conversion must be taken into consideration. When this is done, it is obvious that 1  $\gamma$  of thiochrome is equivalent to 2  $\gamma$  of thiamine. Standards should be replaced every month.

*Recoveries*—When known amounts of thiamine are added to normal urine, the recoveries, as shown in the data of Table II, are quantitative within the limits of the method, ranging from 92 to 101 per cent of the amount added.

Table III presents the data obtained on the recovery of added amounts of thiamine to samples of urine in vitamin B<sub>1</sub> deficiency. It will be observed that the recovery falls considerably below the

theoretical amount added, ranging between 61 and 81 per cent with an average of 75 per cent. It seemed of interest to determine whether this lower yield of thiochrome was due to the fact that the subjects were deficient in vitamin B<sub>1</sub> intake. Two subjects were placed on a very low intake of thiamine and studies were made both of their vitamin excretion into the urine and of the recovery of added thiamine at various intervals for from 22 to 28 days. The data are presented in Table IV. It will be observed that, whereas the recoveries of added amounts of thiamine were approximately theoretical early in the experiment, as avitaminosis B<sub>1</sub> proceeded

TABLE III

*Recovery of Added Thiamine Hydrochloride from Vitamin B<sub>1</sub>-Deficient Urine  
Based on Theoretical Conversion of Thiamine to Thiochrome  
0.077  $\gamma$  of thiamine added as thiochrome.*

Thiamine hydrochloride added	Thiamine hydrochloride added expressed as thiochrome*	Total added thiochrome	Added thiamine found as thiochrome	Recovery†
$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
0.25	0.19	0.154	0.077	61
0.50	0.38	0.269	0.192	77
1.00	0.77	0.462	0.385	77
1.50	1.16	0.654	0.577	77
2.00	1.54	0.854	0.777	77
3.00	2.31	1.320	1.243	81

\* 1  $\gamma$  of thiamine hydrochloride equals 0.77  $\gamma$  of thiochrome.

† With a conversion factor of 65 per cent.

the recovery of added amounts of vitamin B<sub>1</sub> sharply decreased, until at the 28th day in one individual only 77 per cent was recovered and in a second individual on the 22nd day only 80 per cent was recovered. It would appear therefore that the more deficient a subject is, the closer the recovery of vitamin B<sub>1</sub> added to the urine approaches 75 per cent.

It was important to determine whether this lower recovery was due to a failure of conversion of thiamine to thiochrome or to the effect of the presence of the many non-fluorescent substances which are known to increase in the urine of patients suffering from vitamin B<sub>1</sub> deficiency. An experiment was devised to throw some light on this subject. To a pathological urine containing very

small amounts of thiamine hydrochloride, known amounts of the vitamin were added. As will be seen in Table V, the recoveries

TABLE IV

*Effect of Low Thiamine Intake on Excretion of Thiamine in Urine and on the Recovery of Added Thiamine*

0.77  $\gamma$  of thiamine added to sample as thiochrome.\*

Days of diet	Total excretion of thiamine	Thiochrome* in urine sample	Thiamine as thiochrome found*	Added thiamine* in urine as thiochrome	Added thiamine recovered†
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
Subject B. 2	70	0.19	0.67	0.48	95
8	5.2	0.07	0.48	0.41	81
20	Trace	None	0.38	0.38	77
28	"	"	0.37	0.37	77
Subject E. 4	65	0.38	0.88	0.50	100
7	23	0.07	0.56	0.49	98
12	15	0.03	0.46	0.43	86
19	6	0.03	0.44	0.41	82
22	6	Trace	0.40	0.40	80

\* 1  $\gamma$  of thiamine hydrochloride equals 0.77  $\gamma$  of thiochrome.

† With a conversion factor of 65 per cent.

TABLE V

*Recovery of Thiamine Added to Pathological Urine and to the Eluate after Decalso*

0.05  $\gamma$  of thiamine hydrochloride present in urine, expressed as thiochrome.\*

Thiamine hydrochloride added, expressed as thiochrome* at beginning of procedure	Thiamine hydrochloride added found as thiochrome*	Recovery of added thiamine†	Thiamine hydrochloride added to eluate, expressed as thiochrome*	Thiamine hydrochloride added found as thiochrome*	Recovery of thiamine added to eluate†
$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	per cent
0.38	0.18	74	0.38	0.24	97
0.77	0.38	77	0.77	0.50	100
			1.16	0.72	98
1.55	0.78	78	1.55	0.97	100

\* 1  $\gamma$  of thiamine equals 0.77  $\gamma$  of thiochrome.

† With a conversion factor of 65 per cent.

of such added amounts of thiamine were markedly reduced. When, however, known amounts of vitamin were added to the eluate from the Decalso adsorption, the recoveries of such added

amounts were quantitative. These results were interpreted to mean that substances were present in urine in vitamin B<sub>1</sub> deficiency which interfered with the adsorption of the vitamin from the urine rather than in the final oxidative reaction. These substances were non-fluorescent, since the preliminary extraction with isobutyl alcohol reduced fluorescent interfering agents to not more than 5 per cent of the total amount of thiamine present. Thus far all attempts to remove these interfering agents have failed. It has been found, however, that the recoveries of added amounts of thiamine to pathological urine seldom fall below 75 per cent. We have been able to correct for the effect of these interfering agents by carrying through the analysis a duplicate urine sample containing preferably 1  $\gamma$  of thiamine hydrochloride and correcting the observed reading on the basis of the amount of thiochrome actually found. Under circumstances where this is not possible, it is suggested that a correction figure based on a 75 per cent recovery may be used without causing an error of greater than 5 to 10 per cent of the true value.

*Preliminary Washings with Isobutyl Alcohol*—Washing the urine prior to adsorption by Decalso insures the elimination of most of the interfering fluorescent substances such as indoleacetic acid, preformed thiochrome, and certain pigments which if permitted to remain in the urine would greatly increase the amount of ferricyanide necessary for oxidation, thereby giving a high urine blank, or, by virtue of their own fluorescence, interfere with the final reading. In addition to isobutyl alcohol, isoamyl alcohol and normal butyl alcohol were tried. When isobutyl alcohol was used, the alcohol layer exhibited a high content of pigment and fluorescence, while the urine layer showed none of these characteristics. With isoamyl alcohol, the amount of fluorescence found in the alcohol layer was similar to that found in the isobutyl alcohol layer, but the urine still showed fluorescence. When normal butyl alcohol was used, the alcohol layer was less fluorescent and the urine layer more so than with either of the other two reagents. It was concluded, therefore, that of the three alcohols studied isobutyl alcohol was much to be preferred.

*Effect of Certain Therapeutic Agents on Yield of Thiamine*—In spite of the washing with isobutyl alcohol, certain therapeutic agents such as quinine and quinidine will impart fluorescence to

urine which will interfere with the final comparison. Ethyl alcohol, when excreted in the urine or added to it, alters the shade of fluorescence. Ascorbic acid also increases the final fluorescence of the sample and renders it more violet, which makes it impossible to compare satisfactorily with the standards. Nicotinic acid in large amounts has been stated to interfere with the reaction (11). As stated above, the presence of metabolites found in thiamine-deficient patients and also the presence of bile or blood in the urine also interfere with the reaction, probably by reducing the effectiveness of the adsorption. These interfering agents, however, can be corrected for by the use of the precautions stated above.

*Elution of Thiamine from Decalso with Potassium Chloride Solution*—When the Decalso adsorbate is shaken well with 2 ml. of potassium chloride, a minimum amount of potassium ferricyanide is required for oxidation. The use of methyl alcohol as an eluting agent has been suggested (1). The basis for the use of this reagent is that it tends to protect the vitamin from overoxidation by an excess of potassium ferricyanide. It has been found, however, that when the elution is carried out as suggested in the present procedure excesses of this reagent are avoided and the necessity for the use of methyl alcohol eliminated. Furthermore, the yield of thiamine, obtained from pure solutions when potassium chloride is used as the eluting agent, is approximately 10 per cent higher than when this substance is omitted. In the absence of potassium chloride, quantitative yields can be obtained only by the addition of much larger amounts of the oxidizing agent.

*Oxidation*—We have found that 1  $\gamma$  of thiamine was oxidized in pure solution by 1.0 mg. of potassium ferricyanide and that the quantity of the oxidizing agent required does not vary greatly with different urines. 0.2 to 0.3 ml. of a 0.5 per cent potassium ferricyanide solution was usually sufficient for complete oxidation. Occasionally in urines with a high thiamine content a few drops more of the reagents were required. Studies made with pure solutions of thiamine hydrochloride and with urines containing known amounts of vitamin showed that for all amounts of thiamine up to 1  $\gamma$  1 mg. of potassium ferricyanide was sufficient. For amounts ranging between 1.5 and 10  $\gamma$  of thiamine 1.5 to 2 mg. of potassium ferricyanide were required.

*Extraction with Isobutyl Alcohol*—It was of course important to

determine whether or not thiochrome produced by the oxidation with ferricyanide could be completely extracted by isobutyl alcohol. Accordingly, known amounts of thiochrome were added to treated urine after the oxidation. The data given in Table VI indicate that such thiochrome was completely extracted by the isobutyl alcohol in the amounts used.

TABLE VI

*Extraction by 5 Ml. of Isobutyl Alcohol of Thiochrome Added after the Oxidation Stage*

0.19  $\gamma$  of thiochrome present in urine sample.

Thiochrome added to oxidized sample	Total thiochrome found	Recovery
$\gamma$	$\gamma$	per cent
0.31	0.50	100
0.38	0.55	98
0.46	0.65	100
0.61	0.70	100
0.77	0.99	102
0.94	1.13	100
1.4	1.59	100
1.6	1.85	105

## SUMMARY

In the present method we have emphasized the importance of a preliminary extraction of the urine sample with isobutyl alcohol. In our experience with methods in which this step is omitted, such as those of Ritsert (6), Jowett (7), Borson (8), Westenbrink and Goudsmit (12), and Ferrebee and Carden (9), the failure to introduce such a preliminary washing results in a false elevation of 25 to 35 per cent when the readings are made in the neighborhood of 1  $\gamma$  of thiamine in the final comparison. The introduction of this washing step, which is attributed to Wang and Harris (10), reduces this error to not more than 5 per cent.

In a comparison of the results by the present method with those by others in the literature it must be remembered that the standards are so prepared or labeled as to include a 65 per cent conversion factor in the oxidation of thiamine to thiochrome. It would appear from our data that the chief cause of low recoveries from

urine when suitable adsorbing and oxidizing agents are used is the fact that only a portion of thiamine can be converted to thiochrome. However, it is again emphasized that as avitaminosis B<sub>1</sub> increases there is a failure to obtain quantitative yields, which cannot be attributed to a failure of conversion of thiamine to thiochrome. In such instances it is advisable to determine the exact loss in the recovery of a known amount of thiamine. This loss is considered to be due to the presence of certain non-fluorescent metabolites whose concentration increases in the urine as avitaminosis continues. We have never noted a fall in recovery below approximately 75 per cent in any pathological urine specimen. The presence of blood or bile will also cause a reduction of the yield of thiochrome to this level but apparently not below it. The action of these interfering agents is presumed to be attributed to the prevention of optimum adsorption of the vitamin by Decalso. The entire procedure has been carefully checked over for other causes of loss. One important consideration was that due to the effect of atmospheric oxygen. Since in the present method the thiamine in the urine is never exposed to alkali except at the very moment when oxidation by ferricyanide is being carried out, one would not expect that losses due to oxidation by the oxygen of air would occur. This particular point was tested by repeating many determinations in an atmosphere of nitrogen. However, such experiments reveal no significant increase in the amount of thiochrome produced. Of course, repeated shaking of thiamine increases the possibilities of intimate contact of thiamine with molecular oxygen. In the present method, shaking has been kept to the minimum and adsorption carried out by rapid filtration. These steps in the procedure would appear to minimize any losses due to these causes. The replacement of other eluents by potassium chloride has resulted in a much better removal of thiamine from the Decalso and has minimized the amount of potassium ferricyanide required, thereby preventing any danger of over-oxidation of the vitamin.

The method has been found applicable to the determination of quantities of thiamine varying from 0.2 to 2.0  $\gamma$  in 5 ml. of isobutyl alcohol in the final comparison. With dilutions higher than 1:20,000,000 of thiochrome, however, an error of about 15 per cent is introduced into the standard readings. Within the stated



range, differences of the order of 0.1  $\gamma$  are detectable when either the Wood light or the Cohen fluorometer is used. Although the latter instrument possesses greater precision and accuracy, there are, nevertheless, advantages in the use of the eye with the Wood light which the fluorometer lacks. Thus the perception of differences in tonality and quality of fluorescence is a function requiring a fineness of judgment which is only possessed by an experienced and an observant technician.

We have found that the use of so called permanent standards such as quinine or quinidine sulfate is unsatisfactory. As a matter of fact, these materials are poorly soluble and unstable in isobutyl alcohol. They are more stable, it is true, in mixtures of ethyl and isobutyl alcohols. Such mixtures, however, are not satisfactory as extracting agents for thiamine in the procedure, and, in addition, the fluorescent properties of quinine and quinidine in mixtures of these alcohols are not comparable to those of thiochrome.

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## A NEW AND HIGHLY SPECIFIC COLORIMETRIC TEST FOR METHIONINE\*

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A number of years ago, while making a study of the behavior of various compounds with the oxidized nitroprusside reagent of Weber (1), Sullivan found that methionine, 0.2 mg. per cc., gave with this reagent a strong yellow color which after 5 to 10 minutes standing, became a strong red on acidification. Since most compounds react to give color with sodium nitroprusside only in the presence of alkali, the development of a strong red color on the addition of acid suggested the use of the reaction as a distinctive test for methionine. Subsequently unoxidized sodium nitroprusside was found satisfactory, as described by Sullivan and McCarthy (2).

Application of the nitroprusside reaction to a casein hydrolysate indicated that the particular preparation obtained from Dr. D. Breese Jones and made according to the procedure of Van Slyke and Baker (3) contained 3.2 per cent methionine, practically the same amount as was found by Baernstein (4) by an entirely different method.

However, on further work with pure amino acids and amines, various other compounds were found to react more or less like methionine if the degree of alkalization was low. Thus, histidine, histamine, and carnosine, at levels of 1 mg. per cc., reacted somewhat like methionine under the conditions outlined when relatively small amounts of alkali were employed. Further, on standing 30 minutes or longer in the acid solution, tryptophane, 1 mg. per cc., became reddish brown. The tryptophane color, however, was extractable by butyl alcohol and the aqueous layer was pale

\* The data in this paper were taken in part from the dissertation presented by Timothy E. McCarthy in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Georgetown University.

yellow. On the other hand, the red color given by methionine and histidine was not extracted by butyl alcohol.

Despite the interference by histidine, etc., it seemed desirable to develop the reaction as a test for methionine in proteins. Amino acids as a rule are determined in proteins after digestion with acid, whereby tryptophane is destroyed. The seriously interfering substance is histidine if present in large amounts. Some proteins, such as globin, contain much histidine; so an endeavor was made to devise a procedure wherein the interference of histidine would be at a minimum and the positive reaction of substances such as carnosine, histamine, etc., would be eliminated. The following procedure was finally found satisfactory for the determination of methionine, even when relatively large amounts of histidine or other iminazoles were also present.

✓ *Quantitative Procedure for Methionine*—Introduce 5 cc. of the solution to be tested into a 25 × 2.5 cm. test-tube and add 1 cc. of 14.3 N NaOH (57.2 gm. of NaOH in water, diluted to 100 cc.), 1 cc. of a 1 per cent aqueous solution of glycine, and 0.3 cc. of a 10 per cent aqueous solution of sodium nitroprusside, with mixing after each addition. Place the tube in a water bath at a temperature of 35–40° for 5 to 10 minutes. Then cool in ice water for 2 minutes and add 5 cc. of an HCl-H<sub>3</sub>PO<sub>4</sub> mixture (9 volumes of concentrated HCl and 1 volume of 85 per cent H<sub>3</sub>PO<sub>4</sub>) with shaking during the addition of the acid. Continue to shake well for 1 minute and cool in water at room temperature for 5 to 10 minutes. Match against a standard solution of methionine similarly treated.

With the use of the strong NaOH plus the glycine histidine gives no color. The mixture of HCl and H<sub>3</sub>PO<sub>4</sub> gives a clearer color than the HCl alone.

The reaction for methionine has a high degree of specificity. It is negative with all other amino acids found in acid hydrolysates of protein. Methionine sulfoxide is negative and so are homocystine, cystine, and cysteine, but glycylmethionine is positive. Commercial leucine which contains methionine is positive, but purified leucine is negative.<sup>1</sup> If the solution is kept cold at the

<sup>1</sup> The leucine was converted to the methyl ester and purified by the procedure of Abderhalden and Spinner (5). This ester was hydrolyzed with 20 per cent HCl and the solution was neutralized with 5 N NaOH added dropwise with stirring to pH 6.0. On evaporation to incipient crystallization, cooling, and filtering, leucine free from methionine was obtained.

time the acid is added, there is no color reaction given by tryptophane even if it is present in considerable amount.

The methionine reaction is sensitive to 50 parts per million in the Duboscq colorimeter and to 20 p.p.m. in the Klett-Summerson photoelectric colorimeter with use of a No. 54 filter.

*Deviation from Beer's Law*—Owing to the color of the reagent blank, the values obtained by the visual colorimeter do not follow Beer's law accurately. Over a range of from 300 to 540 p.p.m., the average deviation was +9 per cent. The values found with the Klett-Summerson photoelectric colorimeter, however, are quite satisfactory, since Beer's law is followed with a maximum deviation of  $\pm 8$  per cent with concentrations of methionine varying from 25 to 200 p.p.m.

*Variation of Color Formation with Temperature and Time of Heating*—The same amount of color is given over a range of temperature of 35–55° and from 5 to 60 minutes warming time. At least 5 minutes standing at alkaline reaction is necessary, and less color is obtained if the temperature is below 35°.

*Reaction of Methionine with Iron Compounds*—Kolb and Toennies (6) found that methionine in HCl gives a color reaction with cupric chloride. Dr. Toennies in a private communication suggested that the color formed in the nitroprusside reaction might be due similarly to a ferrous or ferric complex of methionine. However, no red color developed upon the substitution of ferrous sulfate, ferric chloride, sodium ferrocyanide, or sodium ferricyanide for nitroprusside.

*Recovery of Methionine Added to Amino Acid Mixture*—To test the validity of the reaction for methionine, a mixture was made of glycine 38, alanine 36, serine 3, isoleucine 209, phenylalanine 31, tyrosine 45, cystine 10, tryptophane 15, proline 41, hydroxyproline 20, aspartic acid 102, glutamic acid 192, histidine 21, arginine 158, lysine 22, ammonia 23 mg. Since the leucine available contained some methionine, it was replaced by isoleucine. The amino acids were dissolved in 75 cc. of 0.1 N HCl. The solution gave a negative methionine reaction. Then to 50 cc. of the solution were added 15 mg. of methionine and the amount of methionine in 5 cc. aliquots was determined. The recovery of the added methionine was 99.6 per cent.

*Application of Test to Determination of Methionine Content of Casein*—Casein samples were prepared by Dr. W. C. Hess of this

laboratory according to the procedure of Van Slyke and Baker (3). The moisture, ash, total sulfur, and cystine contents are given in Table I.

0.5 gm. portions of Samples 1, 2, and 3 were hydrolyzed for 10 hours with 2 cc. of 20 per cent HCl in an oil bath at 125°. The hydrolysates were poured into 100 cc. beakers and the flasks were washed with 2 cc. of water. The solutions were decolorized by

TABLE I

*Moisture, Ash, Total Sulfur, and Cystine Content of Freshly Prepared Casein*

Sample No.	Moisture	Ash	Total sulfur, corrected	Cystine, corrected
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	8.68	0.11	0.87	0.49
2	11.59	0.12	0.88	0.45
3*	0.00	0.12	0.87	0.45

\* Sample 3 is part of Sample 1 dried in a vacuum desiccator over sulfuric acid.

TABLE II

*Methionine Content of Casein and Distribution of Sulfur*

Sample No.	Per cent methionine	Per cent total sulfur		
		Methionine	Cystine	Methionine + cystine
1	2.94	73	15.0	88.0
2	3.64	89	13.6	102.6
3	3.07	76	13.8	89.8
4	3.45	89	10.8	99.8

being warmed with 50 mg. of carboraffin. The carboraffin (carbex E) was washed with 5 cc. of warm N HCl and 5 cc. of cold N HCl. The combined washings and filtrate of each sample were neutralized to pH 3.5 with 5 N NaOH, added dropwise with stirring, and were then diluted with 0.1 N HCl to 50 cc. Methionine was determined in 5 cc. portions with the use of a standard methionine solution, 300 p.p.m. The results, corrected for moisture and ash, are given in Table II.

As tabulated by Toennies (7), the methionine content of casein

determined by the Baernstein method appears to vary from 2.89 to 3.53 per cent. In casein in general, depending on the treatment during preparation, we have found variation of other constituents, cystine in particular. Samples 1 and 3 are practically the same, since Sample 3 was part of Sample 1 dried *in vacuo* over sulfuric acid. Sample 2 was made in the same way as Samples 1 and 3 but was washed with water to a far greater extent and should be a purer sample. Sample 4 was obtained from Dr. D. Breese Jones and was prepared according to the procedure of Van Slyke and Baker. Despite the variation, the methionine sulfur and the cystine sulfur account for most if not all of the total sulfur, in

TABLE III

*Effect of Time of Hydrolysis on Determination of Methionine Content of Casein*

Time of hydrolysis	Per cent methionine, uncorrected for moisture and ash
<i>hrs.</i>	
1	3.09
2	3.05
4	2.92
7	2.90
10	2.90
14	3.12
24	3.00
Average.....	2.99

agreement with the findings of Baernstein (4) and of Kassell and Brand (8) for other casein preparations. In Harris' casein, Baernstein found 3.10 per cent methionine, while Kassell and Brand (9) report 3.06 per cent methionine in Labco casein.

*Effect of Time of Hydrolysis on Methionine Values*—In order to determine the length of time necessary for liberation of the methionine, a grain curd casein was hydrolyzed for varying lengths of time. The methionine values found are summarized in Table III.

It is apparent that the methionine content of casein can be determined as accurately after 1 hour of hydrolysis as after 24 hours. The high and the low of these seven determinations on one sample vary  $\pm 4$  per cent from the average. There is no

evidence of interference produced by the tryptophane, even in the sample hydrolyzed only 1 hour. However, unhydrolyzed protein cannot be used because of the precipitation in the final acidification.

*Methionine Content of Edestin*—By means of a Klett-Summerson photoelectric colorimeter and a No. 54 green filter, the methionine content of a highly purified sample of edestin from hemp-seed was found to be 2.32 per cent, corrected for 9.88 per cent moisture and 0.10 per cent ash. With the Duboscq colorimeter the value was 2.49 per cent. Toennies (7) gives methionine values for edestin, determined by the Baernstein method, of 1.35 to 2.39 per cent. Kassell and Brand (9), using a modified Baernstein method, give 2.3 per cent by the estimation of homocysteine or 2.4 per cent by the volatile iodide method.

#### SUMMARY

A new highly specific colorimetric test for methionine is presented, based on the reaction of methionine with sodium nitroprusside in an alkaline medium followed by acidification.

The methionine content of casein and edestin has been determined by this procedure with reproducible values in good agreement with existing data.

The procedure is simple, and appears to give satisfactory values even with brief periods of hydrolysis.

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# FACTORS WHICH GREATLY INCREASE THE ACTIVITY OF THE PHENOLIC HYDROXYL GROUP OF L-TYROSINE\*

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That tyrosine constitutes an integral part of the structure of many enzymes, hormones, and proteins of immunity and plays an important rôle in their physiological action is becoming evident. Thus, the fundamental relations between the phenolic hydroxyl of this amino acid and the physiological properties of pepsin (1-3), pepsinogen (4), insulin (5, 6), and the chorionic gonadotropic (7) and lactogenic hormones (8) have been demonstrated and it is quite possible that the contribution of this group to the essential nature of many other protein or peptide catalysts will also be observed.

Mirsky and Anson (9) have shown that ferricyanide is reduced not only by the sulfhydryl components of protein molecules but also by tyrosine and tryptophane. These authors found that as reductants tyrosine and tryptophane react very slowly and yet their reducing capacity is greater than that of the sulfhydryl groups. It was pointed out that, although the activity of the non-sulfhydryl reducing groups is enhanced by an increase in pH, rise in temperature, and denaturation, the reduction of ferricyanide by these groups ordinarily extends over a period of at least 5 hours.

In an earlier publication (10) we have presented evidence which indicates that the reducing action of the chorionic gonadotropic hormone is greatly intensified in the presence of phosphate and moderate heat. In the absence of these accelerating factors

\* The material in this paper has been presented in part at the meeting of the American Society of Biological Chemists at Chicago, April, 1941.



oxidation of the hormone proceeds very slowly even in the presence of strong oxidants. Since the physiological activity of the hormone, which decreases with oxidation (10), is apparently dependent upon the presence of the phenolic hydroxyl group of tyrosine (7), it is not surprising to find that this group has reducing properties similar to those of the hormone. These observations have led to a more detailed study of crystalline tyrosine; and the object of the present paper is to present data which indicate that under certain conditions this amino acid is much more reactive as a reductant than has been previously supposed. It would appear that the normal physiological state should provide these conditions.

#### EXPERIMENTAL

The intensity of the reducing action of crystalline *l*-tyrosine (Eastman) was measured by observing the time required for the reduction of a given amount of an oxidant in a dilute solution. The influence of various accelerating or inhibiting agents upon this reduction time was determined. The 0.001 *N* iodine employed as an oxidant was prepared from potassium iodate, potassium iodide, and hydrochloric acid by combining, in order, 1 cc. of a stock iodate solution containing 3.567 gm. of  $\text{KIO}_3$  per liter, 1 cc. of a stock iodide solution containing 13.835 gm. of KI per liter, 15 cc. of distilled water at 0°, 1 cc. of 2 *N* HCl, and, after thorough mixing, sufficient distilled water at 0° to provide a total volume of 100 cc. This dilute solution was prepared each time just before it was used and was kept in an ice bath at 0°. A stock solution of iodine-potassium iodide which is more commonly used was not employed, since it is important to avoid an excess of potassium iodide. This will be discussed below. The dilute potassium permanganate was prepared by diluting a 0.1 *N* solution.

The oxidation-reduction dyes which may be easily obtained cannot be used as oxidants, since they apparently have  $E_0$  values below that of the phenolic hydroxyl of tyrosine. It may be pointed out that epinephrine and homogentisic acid also have  $E_0$  values above those of the usual oxidation-reduction dyes.

The phosphate buffer mixtures were prepared according to Sørensen's tables. Merck's soluble starch, prepared according to Lintner, was employed as an indicator. In each case 1 cc. of

an exactly 0.2 per cent solution of starch prepared from a single source was used. The total volume of all constituents of a given test was adjusted to 10 cc. unless otherwise indicated.

The phenol color values of solutions were obtained by a modification of Herriott's procedure (11), with the phenol reagent of Folin. Before the colors were developed, the reagents and test solutions were cooled to 0°. After color development with the phenol reagent the solutions were allowed to stand 5 minutes at the same temperature. The phosphate precipitate was dissolved with a minimum of glacial acetic acid and the solution was quickly filtered. The color was matched against simultaneously prepared tyrosine standards within 8 minutes after color development. It was found that this procedure did not decrease the phenol value of unoxidized tyrosine; yet it minimized the reduction of the phenol color reagent by reaction products such as hydriodic acid.

### *Results*

*Agents Which Accelerate the Reaction*—The oxidant most frequently employed was iodine. Being a milder oxidant than potassium permanganate, it has certain advantages in following the kinetics of the reaction. Also the more intense color given by iodine, with starch, in the dilute solutions used is desirable. Ferricyanide was not used in order to avoid turbidity which results when the ferric indicator is added to a solution strongly buffered with phosphate. The use of phosphate buffer is particularly significant, since the addition of this salt to an aqueous solution of tyrosine or phenol greatly accelerates the rate at which these substances react with various oxidants. This is indicated by the data presented in Table I.

It will be seen that in the absence of the buffer a week or more may be required for 1 cc. of 0.001 M tyrosine or phenol completely to reduce 1 cc. of 0.001 N iodine at 25°. In the presence of phosphate not more than 1 or 2 minutes is required. Similar relations were observed at 38°, although the reaction was somewhat more rapid.

It has been found that the substitution of some of the other buffers such as citrate or acetate in place of phosphate gives results similar to those just described. However, in some instances

phosphates show a certain degree of specificity in addition to maintenance of pH. This is particularly true in the reduction of silver described below.

From Table I it is also apparent that the complete decoloration of potassium permanganate by tyrosine or phenol is accelerated by the phosphate in a similar manner, and it is of particular interest to note that phenylalanine does not possess this ability to reduce iodine or permanganate rapidly in the presence of phosphate.

In the presence of this buffer tyrosine readily reduces silver nitrate at room temperature when exposed to light. This can be

TABLE I  
*Influence of Phosphate Ion on Oxidation of Phenol, Tyrosine, and Phenylalanine*

Reductant	Time required for 1 cc. 0.001 M reductant to react with 1 cc. 0.001 N oxidant			
	25°		38°	
	Iodine	KMnO <sub>4</sub>	Iodine	KMnO <sub>4</sub>
Phenol .....	1 wk.	24-72 hrs.	> 24 hrs.	> 24 hrs.
“ + phosphate*....	15 sec.	2.5 min.	5 sec.	60 sec.
Tyrosine .....	> 1 wk.	> 1 wk.	20 hrs.	90 min.
“ + phosphate ..	120 sec.	60 sec.	3 sec.	45 sec.
Phenylalanine .....	> 1 wk.	> 1 wk.	20 hrs.	> 24 hrs.
“ + phos- phate .....	> 1 “	> 1 “	23 “	2.5 “
Phosphate alone .....	> 1 “	> 1 “	1 wk.	> 24 “

\* 0.5 cc. of 1 M Sørensen's phosphate buffer, pH 6.81.

demonstrated by combining 1 cc. of 0.001 M tyrosine, 0.5 cc. of 1 M phosphate buffer, pH 6.8, and an excess of silver nitrate such as 1 cc. of a 10 per cent solution. After standing 5 to 10 minutes in daylight (such as that equivalent to 300 foot-candles) the black metallic silver can be observed mixed with the yellow precipitate of silver phosphate. If sufficient ammonium hydroxide is added to dissolve the silver phosphate, the remaining reduced silver is very striking. In the absence of phosphate this reduction does not take place nor does it occur in the presence of phosphate if pure phenylalanine is substituted for tyrosine.

At pH 5.8 the reduction of silver by tyrosine readily takes place

when the solution is buffered with phosphate but it was not observed when acetate, citrate, or phthalate buffers were employed at the same concentration and pH.

Since phenylalanine differs from tyrosine in that it does not react rapidly with iodine, permanganate, or silver nitrate even in the presence of phosphate, it would appear that the phenolic hydroxyl is the group primarily involved. The reaction of this group with permanganate or silver nitrate is undoubtedly one of simple oxidation-reduction; however, in the reaction with iodine substitution must also be considered. Yet increased ease in substitution would in itself suggest a more active phenolic hydroxyl group as the primary factor.

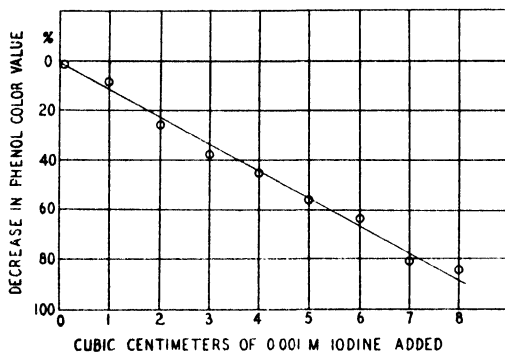


FIG. 1. Decrease in phenol color value of 1 cc. of 0.001 M tyrosine after reaction with iodine.

In order to observe changes in this group which may accompany substitution of iodine in the benzene ring the phenolic color value of tyrosine was followed after the amino acid was allowed to react with various amounts of iodine. From Fig. 1 it will be seen that the phenol color value decreases progressively as the tyrosine reacts with increasing amounts of iodine, suggesting simultaneous oxidation and substitution. While the possibility that the decrease in phenolic color value is due to the stabilizing influence of the substituted iodine has not been excluded, similar decreases in the phenol color value of tyrosine have been observed after the amino acid has been allowed to react with permanganate or silver nitrate in the presence of phosphate. It is of course neces-

sary to avoid the interference caused by the reduced form of the oxidant in each case.

It was found that relatively large amounts of phosphate are required to accelerate to a maximum degree the reaction of tyrosine with iodine. From the data presented in Table II it will be

TABLE II

*Influence of Phosphate Salts on Reaction of l-Tyrosine and Phenol with Iodine*

Weight of salt added*	Time required to react with 3 M proportions of iodine at 38°	
	0.1 mg. <i>l</i> -tyrosine	0.06 mg. phenol
mg.	min.	min.
0	720	390
3.52	577	270
6.85	480	135
10.3	158	30
13.7	20.5	3.25
17.1	8.5	2.0
20.6	6.5	1.5
24.0	5.5	1.5
27.4	5.2	1.5
41.0	4.5	1.5
48.0	4.5	1.5

\* Sørensen's phosphate buffer mixture, pH 5.9.

TABLE III

*Influence of Temperature on Reaction of l-Tyrosine with Iodine*

Temperature	Time required for 0.1 mg. <i>l</i> -tyrosine to react with 3 M proportions of iodine at 38°*
°C.	min.
20	265
30	28.5
40	2.75
50	Instantaneous

\* In the presence of 68 mg. of Sørensen's phosphate salt mixture, pH 5.9.

seen that, at pH 5.9, about 41 mg. of the buffer salt are required for maximum rate of reaction of 1 mg. of tyrosine with 3 M proportions of iodine. Somewhat less phosphate is required at higher pH values.

That moderate heat also exerts a very striking influence on the rate of the reaction is indicated by the data given in Table III.

It will be seen that in the presence of phosphate, pH 5.9, the rate increases about 10 times for each 10° rise in temperature until the reaction becomes instantaneous at 50°. At slightly higher pH values it becomes instantaneous at lower temperatures.

The rate of reaction with iodine is also affected by a change in pH at a constant temperature. Thus from the data presented in Table IV it will be seen that the speed of reaction is approximately inversely proportional to the hydrogen ion concentration. Still further acceleration might be anticipated if the pH were increased to that of the normal physiological range.

The influence of pH and temperature upon the rate of reaction is less evident when permanganate is employed as the oxidant. Also considerably less phosphate is required to bring the reaction

TABLE IV

*Influence of Hydrogen Ion Concentration on Reaction of l-Tyrosine with Iodine*

pH of phosphate buffer mixture*	Time required for 0.08 mg. l-tyrosine to react with 3.6 M proportions of iodine at 38°
	min.
5.90	11.0
6.24	3.1
6.47	1.5
6.64	0.8
6.81	0.5
6.98	0.25

\* 0.5 cc. of 1 M buffer solution was employed in each case.

to its maximum speed. This may be attributed to the stronger oxidizing power of permanganate, making it less dependent upon the favorable conditions which are necessary to support the rapid reaction with iodine.

By keeping all of the variables constant, except the concentration of the reductant, it may be observed that the time required to reduce a given amount of iodine increases exponentially as the concentration of tyrosine decreases.

*Agents Which Retard Reaction with Iodine*—In the presence of phosphate and at elevated temperatures potassium iodide is capable of greatly retarding the reaction of tyrosine with iodine. Therefore it is necessary carefully to standardize the potassium

iodide content of the iodine solution which is to be employed. By preparing the iodine in very dilute solutions from iodate, iodide, and acid as described above it is possible to avoid the marked excess of iodide which is necessary to keep iodine in a more concentrated solution. This is advantageous from the point of view of the present study.

The rate of reaction is also altered by the starch employed as an indicator. The majority of soluble starch preparations retard the reaction somewhat, but some allow it to proceed much more rapidly than others. Although the reason for this is not entirely clear, it should not interfere with the study of the influence of other

TABLE V

*Reduction of Iodine by Casein, Egg Albumin, and Gelatin in Presence of Phosphate Ion*

Weight of protein	Time required to reduce 5 cc. 0.0005 N iodine at 38° *		
	Casein	Egg albumin	Gelatin
mg.	min.	min.	min.
1.0	31	140	
1.25	13.5	74	
1.50	6.5	47	
1.75	4.5	30	
3.0			1140
4.0			480
5.0			300

\* In the presence of 68 mg. of buffer salt, pH 5.9.

factors as long as a uniform amount of starch obtained from a single preparation is employed throughout.

*"Tyrosine Reaction" of Proteins*—It is of interest to compare the reducing properties of various proteins which differ with respect to their tyrosine content. From the results represented in Table V it will be observed that casein, which contains more tyrosine but less cystine than dried commercial egg albumin,<sup>1</sup> reacts with more iodine in the presence of phosphate than does egg albumin. Gelatin containing very little tyrosine shows only

<sup>1</sup> Casein contains 6.6 per cent tyrosine and 0.3 per cent cystine, while egg albumin contains 4.2 per cent tyrosine and 1.3 per cent cystine (from Schmidt (12)).

a slight reaction. If these observations are repeated with the various proteins under the same conditions except that water is substituted for the phosphate buffer solution, only slight activity is apparent even after 96 hours at 38°. These data are apparently in accord with the belief that the reducing capacity of the non-sulfhydryl reducing groups of protein materials is greater than that of the sulfhydryl groups.

It has already been pointed out (9, 13) that the reducing activity of the phenolic groups of common proteins increases as the proteins are denatured. Our experience is in accord with this observation except in the case of some of the more uncommon labile proteins such as gonadotropin (9) which show a decrease rather than an increase in activity after being heated. Preliminary experiments also indicate that a labile component may be associated with serum globulin. For example, while heat denaturation of serum albumin causes about a 25-fold increase in the rate of oxidation of iodine in the presence of phosphate, similar denaturation of the carefully prepared globulin fraction causes a moderate decrease in this activity. It would appear that, in the globulin fraction, the liberation of additional phenolic groups through denaturation is somewhat overshadowed by the oxidation of similar groups which is accelerated by the heat.

#### DISCUSSION

From the data presented it is apparent that while tyrosine is ordinarily oxidized *in vitro* at a very slow rate, even in the presence of relatively strong oxidants, under certain conditions the reaction takes place almost instantaneously. It would appear that the normal physiological environment should provide conditions necessary to support the increased activity of this group, at least in highly specialized proteins which though present in very small quantities have profound metabolic effects.

The fundamental relations between the phenolic hydroxyl group and the physiological activity of various enzymes and hormones have been pointed out by a number of investigators. The chemical activity of this group under physiological conditions is also of considerable interest in immunochemistry. It has been regarded as playing a dominant rôle in determining the immunological character of proteins. The specificity of proteins as an-



tigens can be entirely changed by alterations in the tyrosine groups through nitration or halogenation ortho to the phenolic hydroxyl groups. Yet it has been stated that one outstanding difficulty is to account for the specific activities of antigens in the absence of evidence that the majority contain any specially reactive groups.

In view of the present findings it would appear that this group may not be as stable *in vivo* as was once thought, but it may be one of the active groups being sought. If this is the case, it is quite possible that proteins differ not only in the spatial distribution of these groups but also in the activity patterns which they present, the activity of each group reflecting its molecular environment.

#### SUMMARY

In a study of the reducing action of *l*-tyrosine it was found that the rate of reaction is far more rapid, under certain conditions, than was hitherto supposed. The results of the present study may be summarized as follows:

1. The rate at which tyrosine reacts with iodine, potassium permanganate, and silver nitrate is ordinarily quite slow; however, it may be greatly increased by the addition of phosphate buffer. Small increases in pH greatly intensify the reaction.

2. In the presence of phosphate further marked acceleration results from a moderate increase in temperature, until the reaction becomes instantaneous.

3. This reducing action of *l*-tyrosine may be attributed to the phenolic hydroxyl group.

4. It would appear that the normal physiological state should provide the conditions necessary to support the increased activity of this group. This may further explain why this group is capable of playing such a dominant rôle in the physiological action of various protein catalysts.

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## THE RELATION OF FASTING KETOSIS IN THE RAT TO THE PRECEDING DIET AND THE LIVER FAT

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When rats are fasted they develop a ketonemia (ketosis) and frequently it is high enough to produce a measurable ketonuria. The height of the ketosis is determined by many factors. When the diet is low in choline and in protein, the liver becomes very fatty (1) and the ketonuria on subsequent fasting is much higher (2, 3) than it is in animals that have been on the stock diet or other food mixtures with a higher protein content. It seemed reasonable to assume that the high degree of ketosis during fasting after a low protein intake was incident to the fatty liver. This appeared probable, for ketone bodies arise from the oxidation of fatty acids in the liver, and, during fasting after ordinary diets, fat generally accumulates in the liver during the period in which the ketosis is reaching its ultimate level. However, we have been unable to obtain a good correlation between the degree of ketosis and the amount of fat in the liver and have therefore been led to examine some of the dietary factors which determine the extent of the fasting ketosis.

### *Methods*

The experimental methods are the same as those we have used in other studies. Urine specimens were at first collected under light mineral oil (No. 3, Standard Oil Company of California) to prevent loss of acetone. We now prefer this method for collecting rat urine for almost any purpose. The oil prevents evaporation of the small specimens and loss by wetting of containers during measuring and handling.

Analytical methods for blood ketone bodies (4), urine ketone

bodies (5), urine nitrogen, liver glycogen (6), and liver fat (7) were the usual ones. The liver fat as measured by this method comprised the fatty acids of the neutral fat plus the non-saponifiable lipid material. The blood ketone body values are expressed as acetone.

*Influence of Lipotropic Factors*—Under most dietary conditions choline has a powerful lipotropic effect, tending to prevent the accumulation of fat in the liver and driving out hepatic fat which

TABLE I

*Influence of Choline upon Fasting Ketosis*

*Experiment 1*—Choline was fed during the fasting period. Adult male rats averaging 230 gm. in weight were on Diet 1 (casein 5, Anheuser-Busch's brewers' yeast 5, sucrose 45, Osborne and Mendel's standard salt mixture 5, Crisco 20, butter fat 20) for 10 days and fasted directly from it. Three rats in each group were sacrificed daily for blood ketone determinations. The control group was given 1 cc. of 0.128 N NaCl and the choline-treated group 1 cc. of very nearly neutralized (freshly prepared) 2 per cent choline chloride per sq. dm. of body surface twice daily.

*Experiment 2*—Choline was fed during the period prior to fasting. Adult female rats averaging 203 gm. in weight were on Diet 1 for 10 days. The diet fed the choline group was the same but contained 2 per cent choline hydrochloride. Two rats were sacrificed for the determinations which gave each figure.

Time of fasting, hrs.....		Blood ketone bodies				Liver fat	
		24	48	72	96	0	96
		mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent	per cent
Experiment 1	Control	35	46	28	36		10.6
	Choline	49	46	27	50		5.5
" 2	Control	8	21	22	23	10.7	5.5
	Choline	11	32	37	26	3.4	10.6

has already been deposited (8). But Deuel *et al.* (9) have found that choline administered during the period of fasting does not appreciably affect the ketosis of fasting rats with fatty livers. We have confirmed this observation (Experiment 1, Table I). Neither does the addition of choline to the diet prior to fasting influence the subsequent ketosis (Experiment 2, Table I), although we know that it prevents any accumulation of fat in the liver.

It has been shown that all or most of the lipotropic activity of

protein is due to methionine (10) and that, while this amino acid keeps fat from the liver, cystine is very active in causing the deposition of liver fat (11). From Experiment 3 (Table II) we see that neither of these substances when added to the diet prior to fasting affects the subsequent fasting ketosis. By the end of the 4th day the blood ketone level was of the same magnitude in every case. It should be noted that the blood ketone level before the 4th day was lower in both of those groups (Experiment 3, Groups 7 and 8) which had been receiving a diet of normal pro-

TABLE II

*Influence of Methionine and Cystine upon Fasting Ketosis*

*Experiment 3*—Methionine and cystine were fed prior to fasting. Male rats averaging 251 gm. in weight were on the special diets for 10 days. The *dl*-methionine and *l*-cystine were added to these diets. There were eight rats on each diet and two were sacrificed for blood ketone determinations at the end of every 24 hours of fasting.

Group No.	Diet No.	<i>dl</i> -Methionine	<i>l</i> -Cystine	Blood ketone bodies during fasting			
				24 hrs.	48 hrs.	72 hrs.	96 hrs.
		per cent	per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	1			28	33	33	29
2	1	0.2		21	43	40	29
3	1	0.4		18	37	36	30
4	1	0.7		27	37	42	29
5	1	1.2		29	35	48	26
6	1		1.0	29	35	40	29
7	2			22	28	28	28
8	2		1.0	25	16	26	25

\* The composition of Diet 1 is given in Table I. Diet 2 was the same as Diet 1, but casein and sucrose were changed to 25 parts each.

tein content than in any of the others which had been on a low protein diet.

*Relation of Ketosis to Liver Fat and Glycogen Level*—This led us to follow in detail the development of ketosis during fasting following a low protein intake (Experiment 4, Fig. 1). The rats had not been on this diet long enough for their livers to become fatty and the liver fat concentration increased regularly throughout the period of fasting. But the ketosis as measured by the blood ketone level reached its maximum long before the liver fat at-

tained a very high level. The degree of ketosis shows a more probable causal relation to the liver glycogen level. During fasting the sole source of glucose is from protein catabolism and the glycerol of fat. These rats had been receiving a low protein diet and the amount of "stored" protein available for catabolism must have been very low. This would account for the rapid rise of the blood ketone bodies to their final maximal level. It would

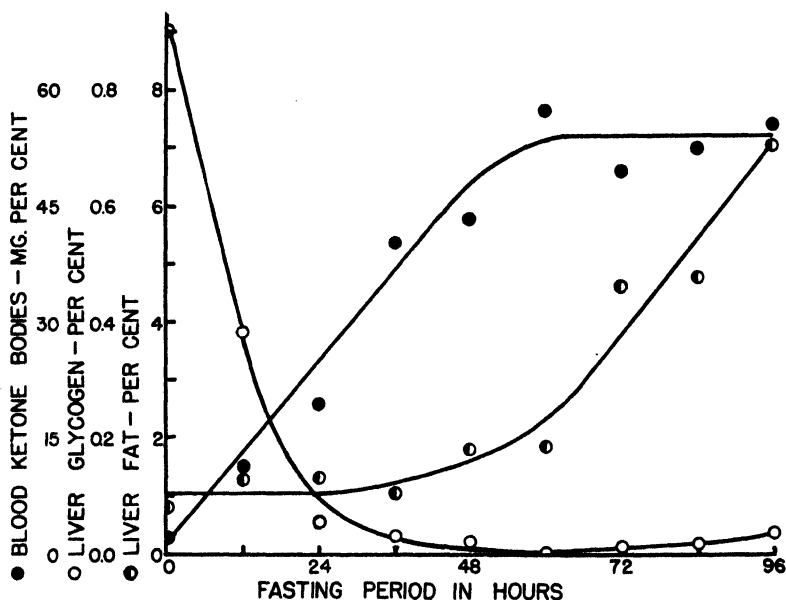


FIG. 1. The relation of fasting ketosis to the changes in liver fat and glycogen. Experiment 4, female rats averaging 184 gm. in weight were on the low protein diet (No. 1) for 8 days before being fasted. Each observation represents the average of determinations made on four animals.

seem probable then that the protein content of the preceding diet is the factor chiefly responsible in determining the rapidity with which the maximum ketosis is reached during fasting as well as the level ultimately attained.

*Influence of Protein Intake in Preceding Diet*—To determine whether or not this is the chief factor governing fasting ketosis, rats were fed protein at various levels. In the first experiments, of which a preliminary report has been made (12), choline was

included in the diets, so that even at the lowest protein level fat would be excluded from the liver. The results showed a very definite influence of the prior protein intake on the subsequent fasting ketosis even when this was measured by the ketonuria. A more convincing experiment has been carried out (Experiment 5, Fig. 2), in which the ketosis was measured by the degree of

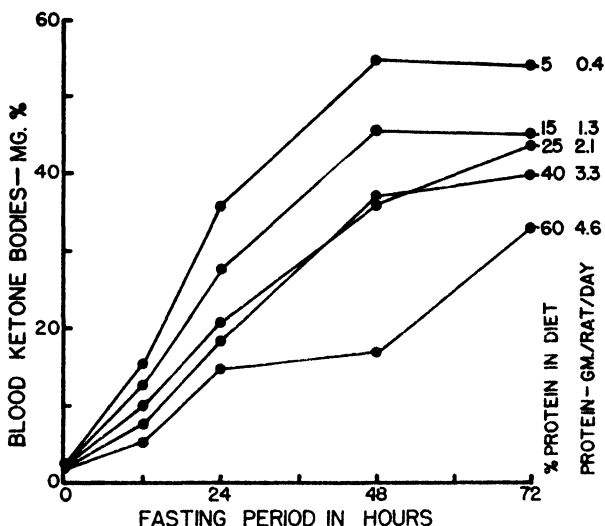


FIG. 2. The development of fasting ketosis in the rat following preceding diets containing variable amounts of protein. Experiment 5, female rats 95 days old and averaging 150 gm. in weight were used. Each observation is an average of determinations made on two rats. The lowest protein diet contained casein 5, sucrose 60, standard salt mixture 5, yeast 5, cod liver oil 5, and Crisco 20. The diets containing larger quantities of protein were similar except that part of the sucrose was replaced by the stated percentage of casein. The animals were on the special diets for 10 days prior to fasting.

ketonemia. The influence of the preceding diet—the less the protein, the greater the ketosis—is quite striking. In our preliminary report (12) we noted that, “The ketosis then might be dependent upon the antiketogenic action of the amount of ‘stored’ protein now available for catabolism. However, nitrogen excretion figures do not support such a supposition.” This last remark was undoubtedly due to our having available only the nitrogen



figures for the first 2 days of fasting and these had not been obtained with high urine volumes, so that the collection error might

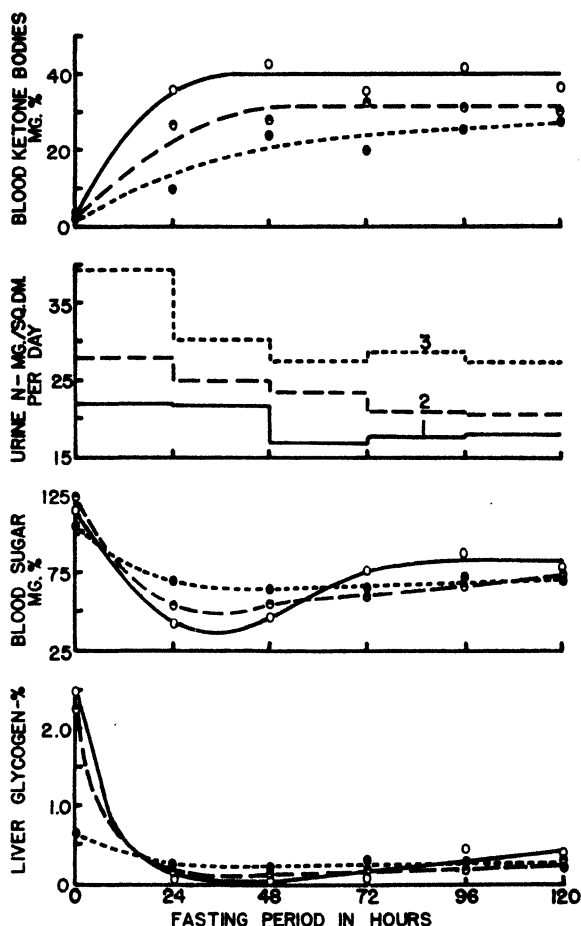


FIG. 3. The relation of fasting ketosis, protein catabolism, blood sugar, and liver glycogen to the protein content of the preceding diet. Experiment 6, each point is an average obtained from observations on three rats. Diet Group 1 ———, low protein diet; diet Group 2 ———, moderate protein diet; diet Group 3 ·····, high protein diet.

be minimized. An experiment was carried out in which careful attention was given to accurate urine collections and with high

urine volumes, so that the urine nitrogen might more nearly represent the end-products of nitrogenous metabolism during the urine collection periods.

In Experiment 6, male rats 100 days old and averaging 241 gm. in weight were fed for 18 days upon special diets and then fasted. All three of the diets were composed of lard 19, Osborne and Mendel's (13) standard salt mixture 5, Anheuser-Busch's high vitamin brewers' yeast 5, cod liver oil 1, and variable amounts of casein and sucrose. Diet 1 contained casein 5 and sucrose 65. Diets 2 and 3 contained casein 25 and 55 respectively, and correspondingly less sucrose. For diet Groups 1, 2, and 3 the average body weights at the end of the feeding period were 237, 269, and 250 gm., while the average food intakes per rat per day were 8.6, 9.4, and 7.9 gm. respectively. Eighteen rats were fed each diet and three from each diet group were sacrificed at the beginning of the fasting period and at the end of every 24 hours thereafter for 5 days. Each rat was given 5 cc. of water by stomach tube twice daily to insure good urine volumes. The results are summarized in Fig. 3. They show very clearly the dependence of the protein ("stored protein") catabolized during fasting—measured by the urine nitrogen excretion—upon the height of the prior protein intake as varied by the protein content of the previous diet. The ketosis, measured by the level of ketone bodies in the blood, bears an inverse relation to the degree of fasting protein catabolism. This is presumably due to the production of antiketogenic material (*i.e.* glucose) from the protein, thus reducing the need for ketone bodies. Excellent support for this view comes from the better maintenance during fasting of the blood sugar as well as the liver glycogen level in those rats (Fig. 3, diet Group 3) which had been receiving the most protein prior to fasting and which catabolized the most during fasting. We have noted the higher fasting liver glycogen after a high protein diet before (14).

#### SUMMARY

Neither the liver fat content *per se* nor any of the agents such as choline, methionine, or cystine which are known to influence the amount of fat in the liver has a significant effect upon the degree of fasting ketosis in the rat.

The rapidity of onset and the degree of ketosis reached during fasting bears an inverse relation to the protein content of the preceding diet. This fasting ketosis is apparently related to the protein intake preceding the fasting period because the latter determines the amount of ("stored") protein available for catabolism during fasting. This serves as a source of antiketogenic material and fasting rats, previously on a high protein intake, better maintain their liver glycogen and blood sugar levels as well as have a lower level of blood ketone bodies.

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## KETOGENIC ACTION OF BRANCHED CHAIN FATTY ACIDS

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Interest in the mechanism of the mode of oxidation of fatty acids wherein the acetone bodies may be formed has caused numerous investigators to be concerned with the ketogenic action of branched chain fatty acids. Baer and Blum (1) fed a series of branched chain fatty acids to diabetics and found that  $\alpha$ - and  $\beta$ -methylbutyric acids as well as  $\alpha$ - and  $\beta$ -ethylbutyric acids led to an increased excretion of  $\beta$ -hydroxybutyric acid in the urine. The inherent difficulties in maintaining any kind of constancy in the basic ketosis tend to vitiate the significance of results obtained in this manner. However, Embden, Salomon, and Schmidt (2) obtained analogous results by liver perfusion studies, but, here again, the method of study offers many difficulties. Wirth (3), perfusing the liver with  $\alpha$ -methylbutyric acid, also found an increase in the formation of ketone bodies. These experiments led to the conclusion (4) that acids with branched chains undergo dealkylation and are then catabolized in the same way (successive  $\beta$ -oxidation) as the corresponding unbranched acid. Later experiments (5) have not supported this dealkylation scheme. Lang and Adickes (6) have recently failed to obtain ketone bodies from the action of liver slices on  $\alpha$ -methylated fatty acids. The availability of a new method for determining ketogenic action offered the possibility of reexamining the ketogenic action of branched chain fatty acids. It has been found that with adequate liver glycogen stores and even with simultaneous carbohydrate feeding the shorter chain fatty acids, of both even (7) and odd numbered (8) carbon atoms, may give rise to ketone bodies. This is useful, for under such conditions there is no reason to believe that ketone bodies would be formed from endogenous fatty acids and thus

cloud the picture. Presumably the short chain acids cannot be stored as such and so are immediately oxidized in whole or part and form ketone bodies, perhaps a preferential method for their utilization under such conditions. The ketogenic action of a series of methyl- and ethyl-substituted fatty acids has been examined by the new method.

#### EXPERIMENTAL

The branched chain fatty acids were prepared by the malonic ester synthesis. They were purified by vacuum distillation of their methyl or ethyl esters in an efficient fractionating column. The compounds were identified by their boiling points and equivalent weights. The fatty acids were injected as solutions of their sodium salts (pH 7.4). The solution flowed from a burette, through an adequate warming device (37°), into the marginal ear vein of the rabbit. The usual procedure was to inject 75 cc. of the solution at a constant rate which required a period of about 45 minutes. Numerous experiments had to be discarded, owing to obvious toxic effects of the injected fatty acids at higher concentrations. Arterial blood samples were drawn from the heart at the start of each experiment and at varying intervals thereafter. The blood ketone concentration was determined by the method of Barnes and Wick (9). At the end of the experiment the animals were injected intravenously with a fatal dose of pentobarbital sodium, and the liver glycogen concentration determined by the method of Good, Kramer, and Somogyi (10). Typical experiments have been presented in Fig. 1 and Table I. Each experiment was repeated two to four times, usually with different doses of the fatty acid. All experiments with a given fatty acid gave analogous results.

#### *Results*

Table I contains a summary of the present results (plus or minus) compared with those obtained by other workers. They fail to agree in large part with those of the earlier workers. In only one case are they not in accord with the more recent results of Lang and Adickes (6) which appear to be the best previous data even though they were obtained with liver slices.

A quantitative comparison of the ketogenic action of the

branched chain fatty acids was not attempted. It is doubtful that the ketone-producing fatty acids form ketone bodies at the same rate. In addition the rate of utilization of the ketone bodies is dependent on their blood level (11).

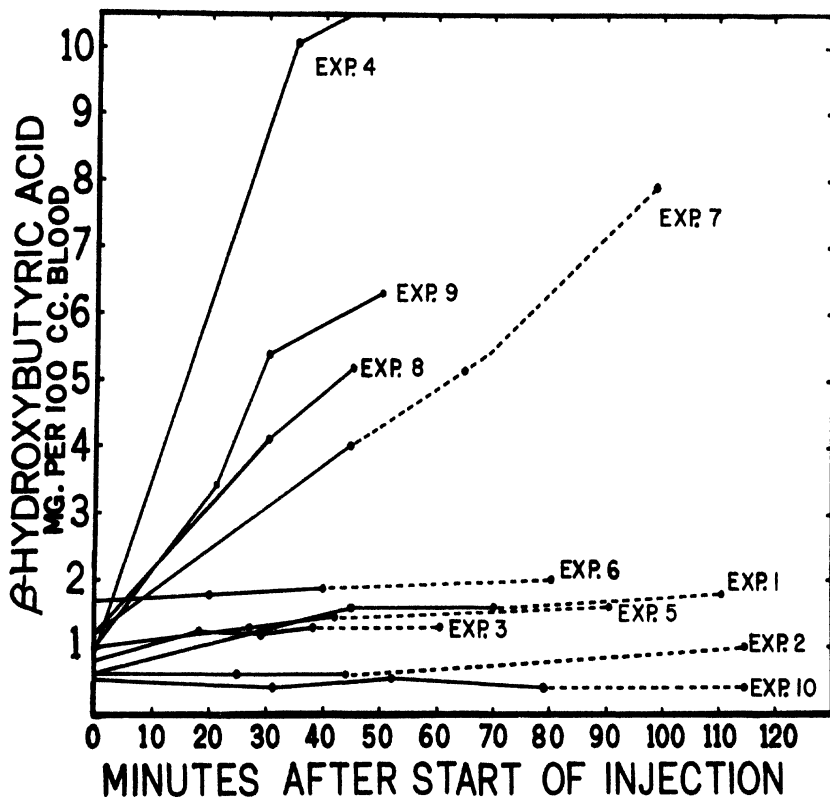


FIG. 1. The influence of the intravenous administration of solutions of the sodium salts of branched chain fatty acids upon the level of ketone bodies in the blood.

#### DISCUSSION

Ketone body formation by the oxidation of fatty acids has been explained in three different ways. The first is the successive  $\beta$  oxidation hypothesis in which 1 molecule of fatty acid furnishes 1 molecule of acetoacetic or  $\beta$ -hydroxybutyric acid. Recently

TABLE I  
*Influence of Fatty Acids on Blood Ketones and Liver Glycogen*

Experiment No.	Weight of rabbit	Fatty acid injected	Liver glycogen at end of experiment	Ketone body formation				
				Wick	Lang-Adickes (6)	Baer-Blum (1)	Embsen-Salomon-Schmidt (2)	
	gm.		mm per kg.	per cent				
1	2000	Isobutyric $\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CHCOOH} \\ \diagup \\ \text{CH}_3 \end{array}$	19.5	5.4	—	—	+	—
2	2990	$\alpha$ -Methylbutyric $\begin{array}{c} \text{CH}_3\text{CH}_2\text{CHCOOH} \\   \\ \text{CH}_3 \end{array}$	29.4	2.3	—	—	+	+
3	2130	$\alpha$ -Ethylbutyric $\begin{array}{c} \text{CH}_3\text{CH}_2\text{CHCOOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	10.5	7.3	—		+	
4	2175	$\beta$ -Methylbutyric $\begin{array}{c} \text{CH}_3\text{CHCH}_2\text{COOH} \\   \\ \text{CH}_3 \end{array}$	19.8	6.0	+	+	+	+
5	3800	$\alpha$ -Methylvaleric $\begin{array}{c} \text{CH}_3(\text{CH}_2)_2\text{CHCOOH} \\   \\ \text{CH}_3 \end{array}$	11.1	1.3	—		—	
6	1812	$\alpha$ -Ethylvaleric $\begin{array}{c} \text{CH}_3(\text{CH}_2)_2\text{CHCOOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	15.3	9.5	—			
7	2075	$\beta$ -Methylvaleric $\begin{array}{c} \text{CH}_3\text{CH}_2\text{CHCH}_2\text{COOH} \\   \\ \text{CH}_3 \end{array}$	17.3	4.1	+	+		+
8	2100	$\gamma$ -Methylvaleric $\begin{array}{c} \text{CH}_3\text{CH}(\text{CH}_2)_2\text{COOH} \\   \\ \text{CH}_3 \end{array}$	14.0	4.5	+	+		—
9	1800	$\alpha$ -Methylcaproic $\begin{array}{c} \text{CH}_3(\text{CH}_2)_3\text{CHCOOH} \\   \\ \text{CH}_3 \end{array}$	8.2	5.6	+	—		
10	2280	$\alpha$ -Ethylcaproic $\begin{array}{c} \text{CH}_3(\text{CH}_2)_3\text{CHCOOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	6.0	4.7	—	—		

this hypothesis has been modified (12) on the assumption that besides the ketone bodies arising from the 4-carbon residue the pairs of carbon atoms removed by  $\beta$  oxidation may be condensed to acetoacetic acid, one of the ketone bodies. Of late Hurtley's scheme (13) of simultaneous alternate oxidation, now called "multiple alternate oxidation," (14-16) has been revived. There is no positive support for this hypothesis and it is difficult to conceive how this mechanism might apply to the results with branched chain fatty acids which are reported here. Likewise, the original  $\beta$  oxidation hypothesis can hardly explain the formation of ketone bodies by  $\beta$ -methylbutyric,  $\beta$ -methylvaleric, or  $\gamma$ -methylvaleric acids.

The results summarized in Table I dealing with the compounds in the butyric acid and valeric acid series show that only those acids which do not have a methyl or an ethyl group on the  $\alpha$ -carbon form ketone bodies. Dealkylation followed by normal catabolism apparently does not occur or it occurs to such a small extent and so slowly that ketone body formation cannot be detected. Raper's theory (17) for the oxidation of such branched chain fatty acids likewise cannot be reconciled with the data reported here.

The acids with no alkyl group on the  $\alpha$ -carbon atom— $\beta$ -methylbutyric acid,  $\beta$ -methylvaleric acid, and  $\gamma$ -methylvaleric acid—all gave ketone bodies. The most likely explanation in all three cases and practically the only possible one in the case of the last two acids is that the ketone bodies were formed by  $\beta$  oxidation and condensation of the removed pairs of carbon atoms (12). The oxidation of  $\beta$ -methylbutyric acid by  $\beta$  oxidation could conceivably leave the 3-carbon residue in the form of acetone. However, two experiments with this acid showed that only 11 and 14 per cent of the total ketone bodies was in the form of acetone, a percentage similar to that found in normal ketosis (18) in which acetone is believed to arise by the decomposition of acetoacetic acid. It was also once more made certain that acetone is not converted to acetoacetic acid or  $\beta$ -hydroxybutyric acid. A rabbit was injected with acetone until the blood level was 50 mg. per cent. At this time and 2 hours after the injection was stopped all of the ketone bodies in the blood were in the form of acetone. Of the two compounds examined in the caproic acid series  $\alpha$ -methylcaproic acid gave ketone bodies. The results with the  $\alpha$ -methyl and  $\alpha$ -ethyl



butyric and valeric acid compounds make it unlikely that the first 2 carbon atoms of the chain whether removed by  $\beta$  oxidation or singly could participate in ketone body formation. More likely is the origin of the latter from the 4-carbon residue. By analogy with  $\alpha$ -methylbutyric acid possibly none of the first 4 carbon atoms of  $\alpha$ -methylcaproic acid could be expected to partake in the formation of ketone bodies. This leaves the last 2 carbon atoms of the molecule to condense with another similar pair to form acetoacetic acid. The failure of  $\alpha$ -ethylcaproic acid to form acetoacetic acid in the same way may possibly be explained by steric hindrance which is produced by the larger group.

#### SUMMARY

The branched chain fatty acids, isobutyric,  $\alpha$ -methylbutyric,  $\alpha$ -ethylbutyric,  $\alpha$ -methylvaleric, and  $\alpha$ -ethylvaleric acids, failed to form ketone bodies when injected into the rabbit. Those acids of these groups which had no methyl or ethyl group on the  $\alpha$ -carbon atom, namely  $\beta$ -methylbutyric,  $\beta$ -methylvaleric, and  $\gamma$ -methylvaleric acids, formed ketone bodies. It is concluded that dealkylation followed by  $\beta$  oxidation is not the manner in which such branched chain fatty acids are catabolized. The ketone body formers probably gave rise to acetoacetic acid by  $\beta$  oxidation and condensation of the pairs of unblocked carbon atoms.

$\alpha$ -Methylcaproic acid gave rise to ketone bodies, probably from the residual carbon chain left after removal of the first 4 carbon atoms. The similar ethyl compound failed to yield ketone bodies and it must be assumed that this larger alkyl group blocked oxidation.

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# DERIVATIVES OF TOBACCO MOSAIC VIRUS

## I. ACETYL AND PHENYLUREIDO VIRUS\*

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An investigation on the relationship of chemical structure to biological activity possesses a twofold interest in the case of viruses. First of all, one may assay the physiological importance of various functional groups in the molecule by converting these groups into chemically inactive forms and testing the resulting derivatives for infectivity. In addition, when a given derivative is found to be biologically active, one may determine the nature of the virus which is produced in the cells of the host. If the infecting molecules served as exact models for reproduction, one would expect to reisolate the virus derivatives from plants so infected. Furthermore, the disease produced by such an altered virus might be different from the normal disease, since it is known that the nature of a virus disease varies with the strain of the virus and that strains of a virus differ from one another in their chemical properties (1, 2). It is possible, therefore, that one might cause structural changes *in vitro* which would, in effect, correspond to the mutation of a virus. If, on the other hand, the inoculation of the virus derivative resulted in the production of normal virus, it might be concluded that the structural changes were reversed within the cells of the host or that that portion of the molecules involved in the structural change was unimportant and played a subordinate rôle in the reactions of virus reproduction.

It has been shown that the sulfhydryl groups of tobacco mosaic virus can be oxidized with iodine without changing the specific virus activity, but that inoculation of the oxidized virus is followed

\* A preliminary report of the present work has been given (*Science*, **93**, 428 (1941)).

by the production of normal virus (3). Schramm and Müller (4) have reported that the amino groups of tobacco mosaic virus could be completely covered by treatment with ketene or phenyl isocyanate without a decrease in specific virus activity, but it was not determined whether the derivatives were propagated as such in susceptible hosts. Prolonged treatment with ketene resulted in inactivation of the virus. This was suggested to be due to involvement of tyrosine phenolic groups, although no chemical proof was given. In the present study, which was begun before the above work came to our notice, tobacco mosaic virus was acetylated and results in qualitative agreement with those of Schramm and Müller were obtained, although from a quantitative standpoint certain differences were observed. We have therefore extended the investigation, with particular regard to determining the nature of the virus which is propagated in plants which have been infected with derivatives of the virus.

In the first experiments, samples of tobacco mosaic virus in 1 M acetate buffer at pH 5.5 were treated with ketene for varying periods of time up to 4 hours. After each period of acetylation, the virus solutions were dialyzed against distilled water at 4° and the preparations thus obtained were used for chemical and biological tests. The changes in amino nitrogen were followed by the ninhydrin method as applied by Ross and Stanley (5). The decrease in tyrosine plus tryptophane groups was determined with Folin's phenol reagent by Herriott's method (6). Tests for biological activity were carried out on plants of both *Nicotiana glutinosa* and *Phaseolus vulgaris* by the half leaf method as used in this laboratory (7). The results are presented in Table I. In Experiment 1 it may be seen that the amino groups were covered more rapidly than the tyrosine plus tryptophane groups and seemed to reach a more or less constant figure representing 40 to 50 per cent coverage. No significant decrease in virus activity was detectable in any of these preparations. In Experiment 2, in which a more rapid flow of ketene was maintained, the total final coverage of amino groups was not appreciably increased, although an increase in rate of acetylation of tyrosine plus tryptophane groups was manifested. The extent of acetylation of these latter groups, like the former, now appeared to approach a limiting plateau, which in this case corresponded to about 20 per cent

acetylation. The partial inactivation evident in the 120 minute run was apparently due to secondary reactions, since the extent of acetylation of the groups tested was not affected. When a very rapid stream of ketene was employed, as indicated in Experiment 3, the extent of acetylation still could not be forced much beyond 50 per cent coverage of amino groups and 20 per cent coverage of the phenol plus indole groups. The virus derivative resulting from a 30 minute treatment under these vigorous conditions was quite gelatinous in character and when examined in the ultracentrifuge was found to be considerably aggregated. However, since the material was found to possess normal virus

TABLE I  
*Acetylation of Tobacco Mosaic Virus in Acetate Buffer*

Experiment No.	Rate of flow of ketene	Time of acetylation	pH at end (5.5 at start)	NH <sub>2</sub> groups covered	Phenol + indole groups covered	Loss in activity
		<i>min.</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Slow	15	5.5	39	9	0
		30	5.5	46	7	0
		60	5.5	36	12	0
		240	5.0	54	18	0
2	Moderate	10	5.5	53	13	0
		30	5.4	44	19	0
		60	5.3	48	22	0
		120	4.5	45	19	40
3	Rapid	30	4.8	55	21	0
		120	4.5			

activity, it is probable that on dilution for biological assay the aggregated particles were dissociated. The 120 minute period of treatment with the rapid stream of ketene resulted in the conversion of the virus into an insoluble precipitate.

It seemed possible that more complete acetylation of the virus might be obtained if the reactions were carried out at a higher pH. 0.5 M phosphate buffer at pH 8.1 was chosen as a suitable medium in which to carry out such experiments. As shown in Experiment 1 of Table II, 30 minutes treatment with a rapid stream of ketene at the more alkaline reaction gave a preparation of virus only slightly more acetylated than the corresponding preparation in acetate buffer. In physical properties, however, the derivative

thus obtained showed much less tendency towards gel structure formation, and when the treatment with ketene was extended to 120 minutes no precipitation occurred, as was the case in acetate buffer under similar conditions. With the longer treatment, the groups tested were not further acetylated, although the virus was partially inactivated.

Experiments were next carried out in which larger samples of virus were treated with ketene for separate 1 hour intervals. After each period of acetylation, the virus was dialyzed, isolated by ultracentrifugation, and dissolved in phosphate buffer at pH 8.1 for further acetylation. The pH of the phosphate buffer remained above 7 for the first half hour of each period of reaction. By acetylating for the separate periods, therefore, the virus was

TABLE II  
*Acetylation of Tobacco Mosaic Virus in Phosphate Buffer*

Experiment No.	Conditions	Time of acetylation	pH		NH <sub>2</sub> groups covered		Phenol + indole groups covered	Loss in activity
			Start	End	Ninhydrin	Van Slyke		
		<i>min.</i>			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Rapid ketene	30	8.1	6.3	58		21	0
	Small scale	120	8.0	4.5	59		20	40
2	Rapid ketene	60	8.1	5.4	46	64	19	0
	Large scale	120	8.1	5.2	62	75	23	25
		240	8.1	5.2	75	83	22	50

treated for longer periods of time at an alkaline pH. For these experiments, the coverage of amino groups was determined by the Van Slyke nitrous acid method (8) as well as by the ninhydrin method. As may be seen from the data for Experiment 2, Table II, the slight further acetylation obtainable under these conditions was accompanied by some inactivation. The per cent coverage of amino groups as determined by the Van Slyke method was found to be somewhat higher than that obtained by the colorimetric method, indicating some lack of specificity in the latter. Thus, values of 55 to 58 per cent, which represent the maximum coverage without inactivation as determined by the ninhydrin method, correspond to a figure of around 70 per cent coverage as determined by the Van Slyke procedure. It may therefore be concluded

from the data as a whole that not more than 70 per cent of the amino groups of tobacco mosaic virus may be substituted without a concomitant loss of specific virus activity. With regard to the phenol plus indole groups, it may be concluded that at least 20 per cent and possibly more of these groups may be covered without the loss of infectivity of the virus.

The amino nitrogen of nine different samples of untreated virus, as determined by the Van Slyke method, was  $0.13 \pm 0.01$  per cent. The analyses were carried out at  $24^{\circ}$  and digestion with nitrous acid allowed to proceed for 20 minutes. Under similar conditions, Schramm and Müller (4) obtained the value of 0.25 per cent amino nitrogen. In addition, they reported negative ninhydrin and Van Slyke tests for their acetylated virus. Since the authors stated that their virus stock was obtained from this laboratory, it is unlikely that the discrepancies can be explained on the basis of different strains of virus.

In order to determine whether tyrosine or tryptophane or possibly both of these amino acids were being affected by the ketene, analyses with the Folin phenol reagent were also made by the "pH 11 method" of Herriott (6). Herriott found that the chromogenic power of acetylated tyrosine derivatives could be recovered by treatment with alkali at pH 11, whereas that of acetyl tryptophane could not. We have found that the acetylated virus after treatment with NaOH at pH 11 gave with the Folin reagent 97 per cent of the color given by a control of unacetylated virus. It therefore seems probable that a portion of the tyrosine in the virus, but little or none of the tryptophane, was affected by the acetylation. The calculation of the exact extent of acetylation of tyrosine in the molecule was made rather uncertain, however, by the fact that the unacetylated, unhydrolyzed virus was found to yield only 59 per cent of the color produced by an equivalent amount of virus following complete hydrolysis by means of 6 N NaOH.

The amount of acetylation of the virus was also checked by acetic acid determinations on preparations of the derivative. Samples of normal and acetylated virus were hydrolyzed with NaOH according to the directions of Sandor and Tabone (9), the hydrolysates were acidified with an excess of citric acid, and the liberated acetic acid was separated by repeated vacuum distilla-



tion. The procedure was similar to that of Herriott (6) but was carried out on a smaller scale. 100 mg. samples of untreated virus liberated 0.0211 mm of acid, whereas the same sized samples of acetylated virus liberated 0.0323 mm of acid. The difference, or 0.0112 mm of acetic acid, was sufficient to account for the acetylation of 70 per cent of the amino groups of the virus and 20 per cent of the tyrosine phenol groups (10). It cannot be doubted, therefore, that the changes in amino nitrogen and in groups reactive to the phenol reagent which were observed on treatment of the virus with ketene were actually due to the acetylation of these groupings. The data indicate that even native virus may contain some acetyl groupings, although this has not yet been definitely established.

A preparation of acetylated virus in which the amino groups were covered to the extent of 70 per cent and tyrosine plus tryptophane groups to the extent of 20 per cent was inoculated at a dilution of  $10^{-5}$  gm. of protein per cc. into a number of young Turkish tobacco plants. The disease produced in these plants was indistinguishable from that in a group of control plants. After a period of 4 to 5 weeks, the viruses produced in the test and control plants were isolated by differential centrifugation. The yields in the two cases were comparable. The virus isolated from the plants inoculated with the acetylated virus possessed the normal amino nitrogen content and showed the same chromogenic power towards the Folin reagent as did the virus from plants infected with normal virus. Further evidence was thus obtained that infecting virus molecules may not necessarily function as exact patterns for reproduction. However, as in the case of the iodine-oxidized virus (3), the objection might still be raised that the plant cells had transformed the derivative into the normal form before reproduction occurred.

In an effort to obtain a virus derivative less likely to be affected by the plant cells, samples of virus in phosphate buffer at pH 8 were treated with an excess of phenyl isocyanate to yield preparations of phenylureido virus. The data obtained with these derivatives are summarized in Table III. It may be seen that with sufficient time of reaction approximately the same per cent of amino groups was covered in this case as in the acetylation reactions. The coverage of phenolic groups was considerably greater after the

phenyl isocyanate treatment, but as with ketene the indole groups were found to be unaffected. No significant inactivation resulted from the conversion of normal virus to the phenylureido derivative. Furthermore, the disease which this derivative produced in Turkish tobacco plants was indistinguishable from that caused by ordinary virus, and the virus reisolated from the infected plants possessed the amino nitrogen content of untreated virus. When one considers the high stability of the phenylureido linkage at neutral and acid reactions (11) and the relatively foreign nature of such a linkage as compared with acetyl linkages in regard to occurrence in living tissues, one is inclined to discount the possibility that phenylureido virus molecules are hydrolyzed by cells of the host plants. If this conclusion is correct, it would follow that the acetyl virus as well was not necessarily hydrolyzed by

TABLE III  
*Phenylureido Tobacco Mosaic Virus*

Experiment No.	Time	NH <sub>2</sub> groups covered		Phenol + indole groups covered	Loss in activity
		Ninhydrin	Van Slyke		
	<i>min.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	90	25	43	43	0
2	410	50	63	36	0

the plant before its biological activity was exhibited. On the basis of the molecular weight of tobacco mosaic virus (12), it may be calculated that around 3000 amino groups and 2000 to 4000 phenolic groups per virus molecule were covered without loss of infectivity of the virus.

In order to determine whether the treated preparations were chemically uniform or consisted of molecules altered to widely different degrees, tests were made with the ultracentrifuge and the Tiselius electrophoresis apparatus. The homogeneity of the preparations as determined in the ultracentrifuge was not measurably altered by the two types of chemical treatment. However, because of the nature of the chemical changes involved, a more sensitive test was provided by the electrophoretic mobility. The electrophoresis experiments were carried out at pH 7.3 in 0.1 ionic  $K_2HPO_4$ - $KH_2PO_4$ -KCl buffer in which 80 per cent of the

ionic strength was provided by the KCl. Runs were made on the normal virus, on the derivatives, and on mixtures of normal virus with each of the derivatives. Tracings of the Longworth

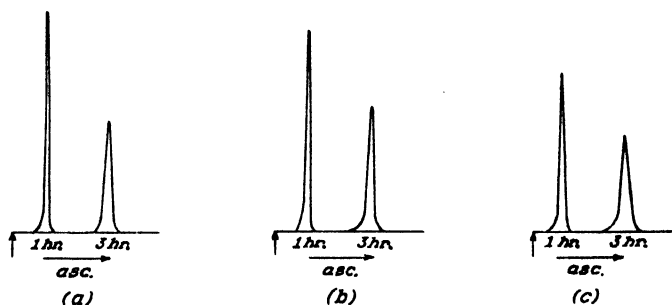


FIG. 1. Tracings of Longworth scanning diagrams of ascending boundaries obtained during electrophoresis of 0.1 per cent tobacco mosaic virus preparations. Field strength, 1.6 volts per cm. The vertical arrows indicate starting positions of boundaries. (a) Acetyl virus; (b) phenylureido virus; (c) normal virus.

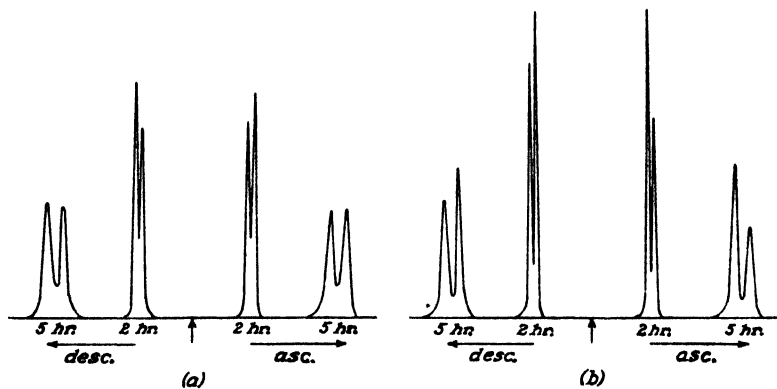


FIG. 2. Tracings of Longworth scanning diagrams of ascending and descending boundaries obtained during electrophoresis of mixtures of 0.1 per cent virus derivatives with 0.1 per cent normal virus. Field strength, 1.6 volts per cm. The vertical arrows indicate starting positions of boundaries. (a) Acetyl and normal virus; (b) phenylureido and normal virus.

scanning diagrams (13) for the various experiments are shown in Figs. 1 and 2. The normal virus possessed a mobility of  $-8.3 \times 10^{-5}$  cm.<sup>2</sup> per volt sec., whereas the derivatives each possessed a

mobility close to  $-9.3 \times 10^{-5}$  cm.<sup>2</sup> per volt sec. It may be seen from the diagrams that the derivatives were quite homogeneous with respect to electrochemical properties and that they could be separated readily from the normal virus when the latter was present. No appreciable amount of unchanged virus was evident in the preparations of the derivatives. The characteristic skewness observed in the scanning curves in certain cases is not indicative of electrochemical inhomogeneity but is the result of a slight streaming of the virus, similar in effect to that observed with vaccinia virus (14).

From the fact that preparations consisting of chemically uniform derivatives of tobacco mosaic virus possessed full biological activity, it must necessarily be concluded that individual units of the derivatives were infective. However, with regard to the question of the propagation of normal virus in plants infected with the derivatives, it might be argued that a very small amount of unchanged virus present in preparations of the derivative could, by virtue of a greater rate of movement or of reproduction within the plant, be solely responsible for the production of normal virus. This possibility was tested by a method based on the findings of Jensen (15) and of Kunkel (16) that, when plants giving the local lesion response were inoculated with a mixture of viruses, any single lesion which was isolated was usually found to contain only one of the viruses inoculated and not a mixture of viruses. Samples of the acetyl derivative of tobacco mosaic virus at concentrations of  $10^{-6}$  to  $10^{-8}$  gm. per cc. were rubbed on leaves of *Nicotiana glutinosa*. Ten single lesions from the infected plants were then removed and each was employed for the inoculation of a batch of young Turkish tobacco plants. For a control, a number of Turkish tobacco plants were simultaneously infected with stock normal virus. After the several inocula were allowed to increase for several weeks, the plants were harvested and the viruses were isolated by the usual procedure. The purified preparations were tested for amino nitrogen by the ninhydrin method and compared with control samples of normal virus. In no case were significant differences observed, and the amino nitrogen in all instances corresponded to that of normal virus. An experiment similar to the one just described was also carried out with the phenylureido virus and identical results were obtained. It has thus been es-

established that the normal virus propagated in plants infected with virus derivatives did not arise from normal virus present as a contaminant in the inoculum, and it may be concluded, therefore, that a large portion of the amino and phenol groups of the virus molecule may be altered without interfering with the basic reactions of virus reproduction.

It is of interest to compare the behavior of the above derivatives of tobacco mosaic virus with the behavior of similar derivatives of other well defined, biologically active proteins. Herriott and Northrop (17) found that with pepsin practically all of the amino groups could be acetylated by means of ketene without the loss of enzymatic activity, but that the progressive coverage of phenolic groups was accompanied by inactivation. Closely parallel results have been reported for insulin (18) and for human chorionic gonadotropin (19). On the other hand, diphtheria toxin (20), the pituitary follicle cell-stimulating, interstitial cell-stimulating, and lactogenic hormones (19, 21), and the gonadotropic hormone in pregnant mare serum (19) appear to require free amino groups for their activity. Gaunt and Wormall (22) found that the treatment of insulin with phenyl isocyanate led to the inactivation of the hormone. In control experiments it was found that the phenolic group of the free amino acid, tyrosine, did not react appreciably with phenyl isocyanate, and it was therefore assumed that the phenol groups of the insulin likewise were unaffected by the reagent. Our findings with tobacco mosaic virus have indicated a marked reaction between phenyl isocyanate and the phenolic groups in the virus protein. In view of the results with the virus and the fact that the acetylamino insulin is biologically active whereas the acetylamino, acetylphenolic insulin is inactive (18), it seems possible that the inactivation of the hormone by the phenyl isocyanate was due to the actual blocking of phenol groups in the molecule.

#### EXPERIMENTAL

*Preparation of Acetyl Virus*—Stock samples of tobacco mosaic virus were obtained by differential centrifugation of the juice expressed from diseased Turkish tobacco plants (23). After the virus was recentrifuged two or three times, it possessed an amino nitrogen content of  $0.13 \pm 0.01$  per cent and was suitable for

acetylation experiments. In the first experiments ketene was passed through 20 cc. of a solution of 0.5 per cent virus in 1 M acetate buffer at pH 5.5. During the acetylation the virus was kept in a cellophane dialysis bag suspended in 1 liter of outside buffer at pH 7.5. A stream of ketene was introduced through a sintered glass filter. The ketene generator and trap for ketene polymers were adapted from the directions of Herriott and Northrop (17) and of Li (24). The acetylations were carried out for periods up to 4 hours. An effort was made to keep the solutions more alkaline than pH 4.8, because when they became too acid the virus was precipitated and inactivated. The temperature was 24°. The virus solutions at the end of reaction were dialyzed at 4° in a rocking apparatus (25) against 40 liters of distilled water. The large scale acetylations were carried out on 180 cc. portions of 0.5 per cent tobacco mosaic virus in 0.5 M potassium phosphate buffer at pH 8.1. In these cases, an outside buffer of 2 liters of 1 M  $K_2HPO_4$  at pH 8.8 was used and was stirred mechanically during the runs. After each 1 hour period of acetylation, during which the pH dropped to 5.4 or 5.2, the virus was dialyzed free of phosphate, isolated by ultracentrifugation, and dissolved in phosphate buffer at pH 8.1 for further acetylation. The hydrogen ion concentrations were determined by means of a glass electrode.

*Preparation of Phenylureido Virus*—In a typical experiment, 100 cc. of 0.1 M phosphate buffer at pH 8.6 and containing 1.63 gm. of ultracentrifugally purified tobacco mosaic virus were cooled by means of an ice bath, and a total of 1.6 cc. of phenyl isocyanate and 7 cc. of 0.2 N NaOH was added with continuous stirring during the course of  $2\frac{1}{2}$  hours. The phenyl isocyanate was added to the cooled solution in 0.1 cc. portions at 10 minute intervals. The hydrogen ion concentration of the solution was maintained between pH 7.9 and 8.5 by the occasional addition of the alkali in 1 cc. portions. The preparation was then allowed to stand at 4° for an additional 260 minutes with occasional stirring. At the end of this time the hydrogen ion concentration was pH 7.5. The precipitated diphenylurea was removed by centrifugation and washed with 80 cc. of 0.1 M phosphate buffer at pH 7. The supernatant liquid, which was similar in appearance to solutions of ordinary tobacco mosaic virus, and the 80 cc. of wash liquid were combined and dialyzed in a rocking apparatus against 40 liters

of cold distilled water during the course of 24 hours. The final preparation contained 1.2 gm. of protein, corresponding to a yield of about 74 per cent based on the starting material.

*Ninhydrin Amino Nitrogen Determinations*—The ninhydrin tests were first carried out by the quantitative procedure used by Ross and Stanley (5). According to this method, 5 to 10 mg. samples of virus in 1 cc. of solution are mixed with 0.3 cc. of pyridine and 0.3 cc. of 2 per cent aqueous ninhydrin and allowed to stand for about 20 hours at 37° for color development to take place. Because of the variability encountered, it was essential to run each determination in triplicate. We have now found that by carrying out the reactions at 65–75° for a period of 30 minutes a much more rapid color development occurs and the variability is greatly reduced. The colored solutions thus obtained were diluted to 8 cc. and compared in the Klett-Summerson photoelectric colorimeter. A green filter No. 54 was used in the estimations. The results of determinations on acetylated virus preparations by the original and by the modified procedures were identical, showing that the heat treatment did not cause further liberation of amino groups. In the absence of pyridine, precipitation of the virus interfered with quantitative estimation. It was found that samples of untreated virus containing less than 2 mg. may yield no color with the ninhydrin reagent, and furthermore that even larger samples of the acetylated virus may give negative results. It was therefore important that sufficiently large samples be employed for the determination. The negative ninhydrin tests reported by Schramm and Müller may have been due to the use of samples of the derivative which were too small.

*Van Slyke Amino Nitrogen Determinations*—Tobacco mosaic virus in sufficiently high concentrations in acetic acid solution yields a stiff gel when sodium nitrite is added. Hence, the manometric procedure of Van Slyke (8), which permits an efficient mixing of reagents, was employed in preference to the volumetric procedure for the amino nitrogen determinations. The analyses were carried out at 24° and digestion with the nitrous acid was allowed to proceed for 20 minutes. At the end of this time, the liberation of nitrogen approached an amount corresponding to  $0.13 \pm 0.01$  per cent amino nitrogen. The slight further liberation of nitrogen which took place almost indefinitely with time of reaction was considered to be of doubtful significance.

*Tyrosine and Tryptophane Determinations*—The extent of acetylation of tyrosine and tryptophane in the virus was followed colorimetrically by means of Folin's phenol reagent, as applied to unhydrolyzed proteins by Herriott (6). For the pH 8 method, samples of 0.5 to 1.0 mg. of virus in 1 cc. of solution were treated successively with 2 cc. of 50 per cent urea, 0.2 cc. of Folin's phenol reagent, and 1 cc. of alkaline phosphate. The alkaline phosphate was made up of 75 parts of 0.4 M  $\text{Na}_2\text{HPO}_4$ , 14 parts of 10 per cent NaOH, and 11 parts of water. The phenol reagent was diluted before use, so that 0.2 cc., together with the other reagents, gave a final pH of 8.2. The urea was essential for the prevention of turbidity. Color intensities were read in the photoelectric colorimeter at some definite time after the solutions had stood for 20 minutes. It was important that all samples in a given determination be measured after exactly the same interval of time because of the fact that, as shown by Herriott, the color intensity increases almost indefinitely under these conditions. For the pH 11 method, 1 cc. samples of virus were treated with 0.1 cc. of 0.2 N NaOH for 15 minutes and then neutralized with 0.1 cc. of 0.2 N HCl before the other reagents were added. For the determination of tyrosine plus tryptophane on hydrolyzed virus, 25 mg. samples of the virus were heated for 6 hours with 1 cc. of 6 N NaOH and diluted to 25 cc. Analyses according to the procedure outlined above were then carried out on 0.74 cc. aliquots neutralized with 0.26 cc. of 1.0 N  $\text{H}_2\text{SO}_4$ . The unknowns were compared with standard solutions containing 0.035 and 0.070 mg. of tyrosine.

*Acetic Acid Determinations*—The hydrolysis method of Sandor and Tabone (9) was employed instead of that of Herriott (6) because of the shorter time required in the former. 100 mg. of virus protein were heated in 2 cc. of 0.5 N NaOH for 8 hours at 100°. The hydrolysate was transferred to a 50 cc. modified Claisen distilling flask, 2 cc. of 1.0 N citric acid and 2 drops of capryl alcohol were added, and the volatile acid which was liberated was distilled *in vacuo* at a bath temperature of about 40°. The distillate was collected in a receiver which was immersed in an ice bath and was titrated with 0.01 N NaOH. Phenolphthalein was used as the indicator. 4 cc. of water and an additional drop of capryl alcohol were added to the residue in the distilling flask and the distillation and titration were repeated. This operation



was carried out six times in all. Under these conditions, controls of 0.6 mg. of acetic acid were practically completely recovered. The blank titration amounted to 0.02 cc. of 0.01 N NaOH. In the case of the hydrolysates of either normal or acetylated virus, traces of volatile acid were liberated almost indefinitely when the distillations were repeated. However, the titration value approached a more or less constant value of 0.10 cc. of 0.01 N NaOH after the sixth distillation. Errors due to the blanks for these titrations were automatically eliminated when the total titration value for the normal virus was subtracted from that for the acetylated virus.

*Activity Measurements*—Determinations of specific virus activity were carried out according to the directions outlined by Loring (7). Both *Nicotiana glutinosa* and *Phaseolus vulgaris* plants were used for the tests. The activity data for each virus preparation were based on results obtained on at least forty half leaves. During the winter months, when lesion counts on *Phaseolus vulgaris* plants kept in the greenhouse tended to be low, the plants were placed in a warm room at 35° for a period of 24 hours after inoculation and then returned to the greenhouse for 2 or 3 days before the lesions were counted. By this procedure, which has been resorted to by Spencer and Price (26), the average lesion count per half leaf was considerably increased.

*Ultracentrifuge and Electrophoresis Measurements*—Determinations of the sedimentation constant and tests for homogeneity with regard to particle size were made in the air-driven type of ultracentrifuge. The measurements were carried out on 0.3 per cent protein in 0.1 M phosphate buffer at pH 7.1.

Electrophoresis experiments were carried out in a Tiselius apparatus equipped with the Longsworth schlieren optical system (13). Preliminary measurements were made with solutions of 0.2 per cent virus protein in 0.1 ionic acetate buffer at pH 5.1. Considerable difficulty in obtaining satisfactory boundaries was encountered at first. It was found that the mechanical movement of the boundary, whether due to the action of the current or the compensating device, caused an apparent streaming of virus particles and distortion of the boundary. This phenomenon went hand in hand with the tendency for gel formation in the solutions. The acetate ion in particular seemed to cause this structure forma-

tion, since, when four-fifths of the acetate concentration was substituted by NaCl, the effect was diminished. The difficulty was largely eliminated when a change was made to 0.1 ionic  $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4\text{-KCl}$  buffer at pH 7.3 in which 80 per cent of the ionic strength was provided by the KCl, and when the boundary was allowed to move at a relatively slow rate of 1 cm. per hour.

#### SUMMARY

Acetyl and phenylureido derivatives of tobacco mosaic virus have been prepared. Ultracentrifuge and electrophoretic measurements carried out on the derivatives indicated a homogeneity comparable to that of the unaltered virus.

Determinations of specific virus activity revealed that around 70 per cent of the amino groups of the virus could be covered with either acetyl or phenylureido groups without significant inactivation of the virus. Further coverage was accompanied by loss of activity. 20 to 40 per cent and possibly more of the phenol plus indole groups also could be covered without inactivation. Primarily the phenol, and not the indole, groups were affected in the above reactions.

When samples of the derivatives were inoculated into Turkish tobacco plants and allowed to propagate, normal virus was formed. It was concluded that 70 per cent of the amino groups and 20 to 40 per cent of the phenol groups in the tobacco mosaic virus molecule are not important to the basic reactions of virus reproduction.

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## THE DIPEPTIDASES OF INTESTINAL MUCOSA\*

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It has long been recognized that intestinal dipeptidase is not an entity. Linderstrøm-Lang (1, 2) showed that at least two dipeptide-hydrolyzing enzymes were present in hog erepsin. One of these, leucylpeptidase, has been investigated more fully (3-5). Leucylpeptidase hydrolyzes both di- and polypeptides, leucylglycine and leucyldiglycine being split at equal rates. It has also been shown (6) that intestinal aminopolypeptidase attacks dipeptides at appreciable rates. Grassmann and his associates (7) have postulated a prolinase to which they attribute the prolylglycine- and prolyldiglycine-hydrolyzing activity of intestinal extracts. It is well recognized that intestinal mucosa contains much dipeptidase activity not due to its content of aminopolypeptidase and leucylpeptidase. As early as 1931 Balls and Köhler (8) were able to obtain preparations which hydrolyzed dipeptides much more rapidly than polypeptides. Recent recognition (9, 10) of the importance of metals and reducing agents in peptidase activity has facilitated renewed study of dipeptidases.

The present investigation represents an attempt to gain some knowledge of the nature of the dipeptidases present in hog intestinal mucosa. Largely because of the instability of these enzymes, efforts to obtain one or more of them in a state of enzymatic homogeneity were unsuccessful. However, the data obtained do give some information regarding the number and activation behavior of the dipeptidases of erepsin.

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## EXPERIMENTAL

*Methods*

*Crude Enzyme*—Fresh hog intestinal mucosa, obtained from a packing plant, was mixed with a little toluene and stored at  $-8^{\circ}$ . After 6 to 7 days portions were thawed. The fairly clear solution which separated was filtered through cheese-cloth and clarified, if necessary, by centrifuging. Solutions prepared in this manner are referred to in the text as crude aqueous erepsin. The dipeptidases are so unstable that much activity is lost before the mucosa can be frozen. Even the frozen preparation is not very stable. However, it was found that fresh mucosa mixed with an equal volume of pure glycerol at the packing plant and stored at  $-8^{\circ}$  retained its dipeptidase activity very well. Maximum activity was obtained after about 10 days and the activity after 50 days was at least as great as on the 1st day. The liquid obtained by centrifuging this mixture to remove the insoluble cellular material will be called crude glycerol extract.

*Peptide Hydrolysis*—The extent of hydrolysis of peptides was determined by the acetone-HCl titration method of Linderstrøm-Lang (11). The details concerning the method as used in this laboratory have been described in a previous publication (9). The concentration of racemic peptides was  $m/15$ , while that of the others was  $m/30$ . All metal activators were present in 0.001  $m$  concentration and the cysteine concentration was 0.003  $m$ . Hydrolysis of peptides was measured after incubation at  $40^{\circ}$ , pH  $8.0 \pm 0.2$ .

The activity values of Tables I to III are expressed as per cent hydrolysis of one optical component of the substrate per minute per cc. of enzyme (1.5 cc. total volume). Although the enzyme concentration and reaction time chosen were always such that the observed hydrolysis was between 20 and 80 per cent, the activities are to be regarded as only approximate, since a linear reaction rate was assumed in their calculation. The reaction time was usually 30 minutes (never more than 4 hours).

*Results*

*Dipeptidases*—In Table I, the effect of a number of activators on the hydrolysis of various substrates by a crude glycerol extract

of intestinal mucosa is shown. Diglycine hydrolysis is activated by cobalt and by manganese. Prolylglycine splitting is activated by manganese and cysteine, but not by cobalt. Alanylglycine splitting is not accelerated by any of the activators tried. Leucylglycine, which is split by leucylpeptidase, is, as would be expected, hydrolyzed more rapidly in the presence of manganese. The whole of the leucylglycine-hydrolyzing activity of the preparation cannot be attributed to leucylpeptidase. As has previously been shown (5), leucylpeptidase splits leucylglycine and leucyldiglycine at equal rates, while leucylglycine is attacked much more rapidly by the crude preparation than is leucyldiglycine. The hydrolysis of *d*-leucylglycine and *dl*-N-methylleucylglycine resembles, in its activation behavior, the hydrolysis of prolylglycine.

If the assumption is made that the activation behavior of a peptidase does not depend on the substrate being hydrolyzed, the data of Table I indicate the presence of at least three dipeptidases: (a) an enzyme requiring no activators, hydrolyzing alanylglycine rapidly, (b) a diglycine-hydrolyzing peptidase, activated by manganese, and maximally activated by cobalt, and (c) a manganese-activated peptidase splitting prolylglycine and probably other substrates. Since the activation behavior of *dl*-prolylglycine, *dl*-N-methylleucylglycine, and *d*-leucylglycine is similar, it might be postulated that they were all split by an enzyme not requiring a free  $\alpha$ -amino group in the *l* position.

In the crude preparation of Table I, a part of the dipeptide-hydrolyzing activity is due to aminopolypeptidase (6) and leucylpeptidase (3, 5). Moreover, the crude material might contain natural activators in sufficient concentration to affect the activation behavior of the various enzymes. It has not been possible to obtain a dipeptidase preparation free of polypeptidase activity. By successive adsorption of the dipeptidases on alumina C $\gamma$  and on  $\beta$ -Fe $_2$ O $_3$ ·H $_2$ O it is possible to remove a large part of the aminopolypeptidase activity and much inactive material. The preparation and properties of such a solution are described in Table II. It will be noted that the activation behavior of dipeptide hydrolysis resembles that of Table I very closely. From the known substrate specificity of aminopolypeptidase (6) it may be calculated that only a negligible proportion of the dipeptide hydrolysis of the preparation is due to this enzyme. The amount of leucylpeptidase

TABLE I

*Activity of Crude Glycerol Extract of Intestinal Mucosa*

The enzyme was 73 days old; its preparation is described under "Methods."

Substrate	Activity*					
	No activator	Cysteine	Mn <sup>++</sup>	Mn <sup>++</sup> -cysteine	Co <sup>++</sup>	Co <sup>++</sup> -cysteine
Diglycine.....	42	14	127	92	162	22
dl-Prolylglycine.....	15	<33	87	93	11	60
dl-Alanylglycine.....	320	175	189	183	211	251
dl-Leucylglycine.....	83	47	107	113	60	77
d-Leucylglycine.....	0.9	1.0	1.4	2.6	1.2	0.9
dl-N-Methylleucylglycine..	1.7	2.3	4.7	11.3	3.7	1.7
dl-Leucyldiglycine.....			51			
Triglycine.....	33					

\* Activity units are defined under "Methods."

TABLE II

*Activity of Partially Purified Dipeptidase Preparation*

A crude glycerol extract (age 48 days) was acidified to pH 5.1 with acetic acid and centrifuged. (Throughout these experiments all pH values were obtained with a glass electrode but values for solutions of high glycerol concentration represent only the apparent pH.) The supernatant liquid was adsorbed with four-fifths of its volume of alumina C<sub>γ</sub> in 50 per cent glycerol (1 cc. of adsorbent was equivalent to 16 mg. of Al<sub>2</sub>O<sub>3</sub>). The adsorbate was washed with 50 per cent glycerol at pH 5.1 and eluted with 50 per cent glycerol for about 30 minutes at pH 8.5 (NaOH). This eluate was acidified to pH 5.1 (acetic acid), readsorbed with three-fifths of its volume of β-Fe<sub>2</sub>O<sub>3</sub>·H<sub>2</sub>O (57 mg. of Fe<sub>2</sub>O<sub>3</sub> per cc. of adsorbent) (12), washed, and eluted as before.

Substrate	Activity					
	No activator	Cys-teine	Mn <sup>++</sup>	Mn <sup>++</sup> -cysteine	Co <sup>++</sup>	Co <sup>++</sup> -cysteine
Diglycine.....	19	8	60	37	87	6.7
dl-Prolylglycine.....	5.3	26	28	39	3.7	18
dl-Alanylglycine.....	131	80	81	79	100	93
dl-Leucylglycine.....	31	25	39	49	26	36
dl-Leucyldiglycine.....			18			
Triglycine.....	4.1					

present would have an appreciable effect only on the hydrolysis of leucylglycine.

The data of Tables I and II show diglycine hydrolysis to be greatly activated by manganese and cobalt ions. Alanylglycine hydrolysis is not activated by these metals. This indicates that if the enzyme hydrolyzing diglycine does attack alanylglycine, its effect is masked by the presence of another alanylglycine-splitting enzyme which is inhibited by these metals. If part or all of this enzyme could be removed, it should be possible to

TABLE III

*Activity of Enriched Diglycine Enzyme*

To crude aqueous erepsin was added 43 per cent of its volume of saturated ammonium sulfate at pH 6.45. The precipitate was dissolved in water, made to pH 8.0, and dialyzed for 1.5 hours at 10° against 0.01 M borate buffer, pH 8.0. At least 1 sq. cm. of cellophane was used per cc. of solution. The enzyme was then precipitated with mineral acid at pH 4.5 and the redissolved precipitate adjusted to pH 8.5 in 50 per cent glycerol.

Substrate	Activity					
	No ac- ticator	Cysteine	Mn <sup>++</sup>	Mn <sup>++</sup> - cysteine	Co <sup>++</sup>	Co <sup>++</sup> - cysteine
Diglycine.....	0.83	1.17	1.33	1.17	3.50	1.83
<i>dl</i> -Prolylglycine.....	0.73	0.73	1.47	0.90	0.60	0.53
<i>dl</i> -Alanylglycine.....	2.8	1.8	3.7	3.2	5.2	3.5
<i>dl</i> -Leucylglycine.....	1.5	0.9	2.3	1.7	2.3	1.0
<i>d</i> -Leucylglycine.....	1.0	0.93	1.47	1.00	0.83	1.0
<i>dl</i> -N-Methylleucylglycine...	1.0	1.0	1.73	0.93	0.87	1.0
<i>dl</i> -Leucyldiglycine.....	3.0	2.5	3.5	2.5	3.8	3.7
Triglycine.....	1.6	1.3	2.0		2.0	

determine whether the cobalt-activated diglycine enzyme attacks alanylglycine. It has been found that the ratio of diglycine activity to alanylglycine activity can be increased by precipitating crude aqueous erepsin with ammonium sulfate and then with mineral acid. Some enzymatic properties of such a preparation are shown in Table III. The hydrolysis of *dl*-alanylglycine and *dl*-leucylglycine is here activated by cobalt. This indicates that they are also attacked by the enzyme responsible for the cobalt-activated diglycine hydrolysis. It may also be seen from Table III that triglycine hydrolysis is increased somewhat in the presence of



manganese and cobalt, which activate diglycine hydrolysis. Since aminopolypeptidase is not activated by these metals (9), this is undoubtedly due to acceleration of the hydrolysis of the diglycine produced when aminopolypeptidase splits triglycine.

It should also be noted that the preparation of Table III splits *d*-leucylglycine almost as rapidly as *dl*-leucylglycine. The three substrates lacking an amino group of *l* configuration, *dl*-prolylglycine, *dl*-N-methylleucylglycine, and *d*-leucylglycine, are all split at approximately the same rate, and are all activated by manganese. Leucylpeptidase, which is also manganese-activated, splits these peptides only very slowly. This strongly suggests the existence of a manganese-activated peptidase not requiring an amino group. It is worthy of note that hydrolysis of the three substrates lacking a free *l*-amino group is, in the crude enzyme (Table I), accelerated more by manganese plus cysteine than by manganese alone. Moreover, in the crude preparation, these three substrates are split at widely differing rates, whereas in the preparation of Table III they are split at approximately equal rates. It is therefore evident that more than one enzyme is able to hydrolyze them.

*Hydrolysis of Prolyl Peptides*—Grassmann, Dyckerhoff, and von Schoenebeck (7) have considered prolylglycine hydrolysis by intestinal enzymes to be due to "prolinase," an enzyme presumably responsible for the hydrolysis of both prolylglycine and prolyldiglycine by intestinal erepsin. They found that crude preparations split prolylglycine rapidly, but that dried preparations did not, although they still possessed "dipeptidase" activity as measured by leucylglycine hydrolysis. At the time their work was done, it was not realized that leucylglycine was split by leucylpeptidase as well as by dipeptidases. The work of Linderstrøm-Lang (1, 2) indicates dried intestinal preparations to be much richer in leucylpeptidase than in other dipeptide-splitting enzymes.

In Table IV the dipeptide-splitting properties of a dried intestinal preparation are shown. It will be noted that by far the most active dipeptide-splitting enzyme in the preparation is leucylpeptidase. Leucyldiglycine is split 25 times as rapidly as diglycine. Prolylglycine, in the absence of an activator, is split very slowly if at all. The data of Grassmann *et al.*, obtained without the addition of metals, correspond to the values in the third column of

Table IV. It is evident that such data, obtained by the use of dried preparations, show only that the leucylpeptidase does not attack prolylglycine rapidly, and are not indicative of the existence of a specific prolinase. The activation data of Tables I to III indicate that crepsin contains a number of dipeptidases, at least one of which attacks prolylglycine. However, there is no evidence for a specific prolinase.

Grassmann *et al.* also attributed the rapid hydrolysis of prolyldiglycine by crude erepsin to "prolinase" rather than to amino-

TABLE IV

*Dipeptide Hydrolysis by Extract of Dried Intestinal Mucosa*

Fresh hog mucosa was washed repeatedly with excess acetone and dried in a vacuum desiccator. The dried material was extracted 3 days with 80 per cent glycerol. 1.5 cc. of reaction mixture contained extract equivalent to 5.0 mg. of dried mucosa.

Substrate	Hydrolysis			Relative activities		
	Time	No MnSO <sub>4</sub>	0.001 M MnSO <sub>4</sub>	Dried mucosa	Leucyl- pepti- dase (5)	Amino- poly- pepti- dase (6)
	hrs.	per cent	per cent			
<i>dl</i> -Leucyldiglycine.....	0.5	22	40	100	100	100
<i>dl</i> -Leucylglycine.....	0.5	24	34	85	100	10
<i>dl</i> -Alanylglycine.....	4.0	34	55	17	9	50
Diglycine.....	4.0	10	14	4	1	20
<i>dl</i> -Prolylglycine.....	4.0	1	16	5	3	20

polypeptidase, since they found that dried preparations were capable of splitting leucyldiglycine but not prolyldiglycine. It has been shown in a previous communication (6) that intestinal aminopolypeptidase splits prolyldiglycine as well as methylated tripeptides (N-methylalanyldiglycine, sarcosyldiglycine) rapidly, and that leucyldiglycine-splitting preparations do not necessarily contain aminopolypeptidase. Abderhalden and Abderhalden (13), however, have questioned our demonstration of the rapid hydrolysis of methylated tripeptides by intestinal aminopolypeptidase. They show that a dried intestinal preparation is unable to hydrolyze sarcosyldiglycine, but conclude that it contains aminopolypeptidase, because leucyldiglycine is rapidly split. It therefore

appears necessary to emphasize once more that leucyldiglycine hydrolysis is not a criterion of aminopolypeptidase activity. In Table V the activity of a dried intestinal mucosa preparation toward a number of tripeptides is shown. It will be noted that its enzymatic properties resemble closely those of purified leucylpeptidase, but are widely different from those of aminopolypeptidase. Furthermore, hydrolysis of all substrates was accelerated by  $\text{MnSO}_4$ . Manganese ions activate leucylpeptidase but not aminopolypeptidase (9). The present dried preparation, and that of Abderhalden, was, like all leucylpeptidase preparations, very inactive toward sarcosyldiglycine. It is evident that Abderhalden and Abderhalden, using an erepsin preparation which was rich in

TABLE V

*Tripeptide Hydrolysis by Extract of Dried Intestinal Mucosa*

The preparation was the same as in Table IV, with additional washing with ether before drying. 1.5 cc. of reaction mixture contained extract equivalent to 2.8 mg. of dried mucosa.

Substrate	Hydrolysis			Relative activities		
	Time	No $\text{MnSO}_4$	0.001 M $\text{MnSO}_4$	Dried mucosa	Leucylpeptidase (5)	Aminopolypeptidase (6)
	hrs.	per cent	per cent			
<i>dl</i> -Leucyldiglycine.....	0.5	18	42	100	100	100
Triglycine.....	4.0	8	11	3	1	600
Sarcosyldiglycine.....	4.0	0	3	1	<1	106
<i>dl</i> -Prolyldiglycine.....	4.0	14	20	6	1	700

leucylpeptidase but contained little or no aminopolypeptidase, concluded that since their dried erepsin did not hydrolyze sarcosyldiglycine, aminopolypeptidase, which is contained in fresh but not dried erepsin, was also incapable of splitting this compound.

The sarcosyldiglycine used in this, and in previous papers, was made by the reaction of chloroacetyldiglycine with ammonia-free aqueous methylamine. It gave a neutral equivalent of 203 (the calculated value) on titration with HCl in 95 per cent acetone.

## SUMMARY

1. Hog intestinal mucosa contains a number of dipeptidases. These enzymes are very unstable in aqueous solution but may be kept for months in 50 per cent glycerol at low temperatures.

2. On the basis of activation studies on various erepsin fractions, three of these dipeptidases may be partially characterized: (a) an alanyl-glycine-splitting enzyme not activated by the substances used, (b) an enzyme hydrolyzing diglycine rapidly, activated somewhat by manganese but best by cobalt ions, and (c) a manganese-activated enzyme hydrolyzing prolyl-glycine and probably other dipeptides not having an amino group in the *l* configuration. Other dipeptidases are undoubtedly present.

3. Most of the peptidase activity of dried intestinal mucosa is shown to be due to leucyl-peptidase.

4. There is no evidence for the existence of a specific prolinase attacking all prolyl peptides.

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# REMOVAL OF PYRUVIC ACID FROM HUMAN BLOOD IN VITRO\*

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The removal of pyruvate *in vitro* from human blood under certain experimental conditions has been adequately demonstrated (1, 2). Wilkins, Weiss, and Taylor (1) concluded from simultaneous studies of bisulfite-binding substances and pyruvic acid levels in the blood that the latter may be converted into other ketone bodies. Dische (3) found that the removal of hexose diphosphate by blood cells was increased by the addition of pyruvate, under which conditions a phosphate ester difficult to hydrolyze (probably phosphoglycerate) was formed. He concluded that the reaction  $\text{pyruvate} + \text{triose phosphate} \rightleftharpoons \text{lactate} + \text{phosphoglycerate}$  described for muscle (4, 5) occurred also in blood.

In this paper we are reporting our studies on the removal of pyruvic acid *in vitro* from human blood.<sup>1</sup>

## EXPERIMENTAL

Blood was drawn from selected patients on the wards of the Psychiatric Division of Bellevue Hospital. The subjects were chosen on the basis of the absence of any somatic disease or mental illness other than psychoneurosis. All specimens were obtained from fasting subjects at rest in bed. Unless otherwise mentioned, coagulation of the blood was prevented by the use of 0.2 per cent potassium oxalate.

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<sup>1</sup> A preliminary report (6) of these studies was presented before the Fifty-third annual meeting of the American Physiological Society at Chicago, April 15-19, 1941, and has since been largely confirmed (7).

The blood was added to an equal volume of a solution of pyruvic acid containing, in 1 ml., 200  $\gamma$  of the acid as sodium pyruvate, plus the other substances tested. No attempt was made to prevent hemolysis. The sodium pyruvate was prepared from three times redistilled pyruvic acid, diluted, and neutralized according to Lu (8). Controls, prepared in exactly the same manner except that pyruvate was omitted, were used in each experiment.

The samples were then incubated at room temperature with frequent agitation. After a predetermined period they were precipitated with 4 times their volume of 10 per cent trichloroacetic acid, except when lactic acid was to be determined. For the determination of lactic acid an aliquot portion of each sample was precipitated according to Somogyi (9). Another aliquot was precipitated with trichloroacetic acid and used for pyruvic acid determinations. The same amount of sodium pyruvate (in 0.1 per cent solution) which had been added to the experimental samples was added to the controls after precipitation, an equal volume of water being added to the experimental samples.

Pyruvic acid was determined in aliquot amounts of the filtrates according to a previously described method (2). The differences between the values found for pyruvic acid in the controls and in the experimental samples represented the amounts of added pyruvic acid removed by the blood.

Lactic acid was determined by the method of Wendel (10). Pyruvic acid, in the concentrations used, does not interfere with lactic acid determinations by this method.

Bisulfite-binding substances were estimated by a modification (11) of the method of Clift and Cook (12), dibasic sodium phosphate being used to release the bound bisulfite instead of sodium bicarbonate.

Glucose was determined in 2 ml. of blood filtrate (0.2 ml. of blood) by a modification (13) of the method of Hagedorn and Jensen, 0.002 instead of 0.005 *N* sodium thiosulfate being used for the final titration.

The evolution of carbon dioxide was measured in the Warburg apparatus. 1 ml. of blood and 0.8 ml. of phosphate buffer (pH 7.4, 0.2 molar) were placed in the main compartment of a vessel with two side arms. 0.2 ml. of a 0.1 per cent solution of pyruvic acid as sodium pyruvate (pH about 7.4) was placed in one arm,

and 0.2 ml. of 0.8 N sulfuric acid in the other arm. The control flasks contained 0.2 ml. of water in the first side arm in place of the pyruvate. The flasks were shaken at 30° in an atmosphere of nitrogen until spontaneous gas production was minimal, at which time the pyruvate (or water) was tipped into the main compartment. Again when there was no further evolution of gas (usually after 1 hour) the acid was tipped into the mixture from the other arm. Incubation was continued until all gas production ceased. The amount of gas evolved from the time of the addition of the pyruvate (or water) until the end of the experimental period was recorded. The difference in gas production between the flasks containing pyruvate and the corresponding control flasks containing water represented the carbon dioxide evolution due to decarboxylation of pyruvate.

Aliquot portions of blood were incubated in the same manner except that they were removed for the determination of pyruvate before the addition of acid.

All determinations were done in duplicate.

### Results

The ability of oxalated whole blood to remove added pyruvate is increased by allowing the blood to stand for 30 to 60 minutes at room temperature before the addition of the pyruvate (Table I). Standing for a longer period of time does not further increase the rate of pyruvate removal. There may even be a slight decrease when the blood is permitted to stand for 24 hours.

The data in Table II show that the rate of pyruvate removal decreases as the period of incubation with pyruvate is prolonged. The amount of pyruvate removed per ml. of blood is constant, regardless of the total amount of blood used.

The removal of pyruvate from blood, *in vitro*, is influenced by a number of factors and substances. It is increased by phosphate in a concentration of 0.075 M (pH optimum 7.4) (Table I). Sodium cyanide (neutralized to litmus with dilute sulfuric acid) increased the disappearance of pyruvate regardless of whether the pyruvate was incubated with blood or with distilled water alone. It has been reported previously (2) that this action of sodium cyanide is not inhibited by concentrations of iodoacetate which completely stop pyruvate degradation in blood. The addition of pyruvic



acid cyanohydrin (14) to blood did not cause any significant formation of lactic acid. The increased pyruvate removal caused by

TABLE I

*Effect of Various Factors upon Pyruvate Removal from Human Blood*

Anticoagulant	Period of incubation of blood before addition of pyruvate	Phosphate buffer concentration (pH 7.4)*	Sodium fluoride concentration*	Pyruvic acid removed in 30 min. by 1 ml. blood			
				$\gamma$	$\gamma$	$\gamma$	$\gamma$
0.2% oxalate	5 min.	M	per cent	19	29	25	
	30 "			45	50		
	60 "			45	52	57	
	180 "			42	50	48	
	24 hrs.			38		44	
0.2% oxalate	60 min.	0.075		45	57	69	48
	60 "			81	120	138	88
0.2% oxalate	60 min.	0.075	0	146	89	131	155
	60 "	0.075	2	88	49	69	40
25 mg. % heparin	60 "	0.075	0		28	61	58
	60 "	0.075	2		25	46	28

\* Phosphate and fluoride were added 1 hour after withdrawal of the blood from the vein.

TABLE II

*Rate of Pyruvate Removal from Oxalated Whole Blood Incubated for 60 Minutes, at Room Temperature, before Addition of Pyruvate*

Time of incubation with pyruvate	Experiment 1		Experiment 2	
	Total removed	Pyruvate removed per min.	Total removed	Pyruvate removed per min.
min.	$\gamma$	$\gamma$	$\gamma$	$\gamma$
0.75			5	6.7
3.00			12	4.0
10.00			32	3.2
15.00	28	1.9		
30.00	41	1.4	46	1.5
60.00	59	1.0	61	1.0
120.00	67	0.6		
136.00			77	0.6

cyanide is therefore not due to an activation of an enzyme system but to the formation of pyruvic acid cyanohydrin.

It has previously been shown that pyruvate removal is inhibited

by iodoacetate in a concentration of 0.2 per cent (2). In addition, sodium fluoride in a concentration of 2 per cent was found definitely to decrease pyruvate removal (Table I). Concentrations of 1 per cent or less had no appreciable effect.

An apparent decrease in pyruvate removal occurs when defibrinated or heparinized bloods are used instead of oxalated blood (Tables I and III).

The addition of thiamine and cocarboxylase to normal human blood *in vitro* had no effect upon the rate of removal of pyruvate. This was pointed out in our preliminary report (6) and constitutes evidence against the possibility (2) that cocarboxylase might be involved in the removal *in vitro* of pyruvate from blood.

The removal of pyruvate did not significantly vary when the blood was incubated at temperatures ranging from 25–37°. Nor was there any consistent variation in the amount of pyruvate removed according to whether the blood-pyruvate mixture was incubated in an atmosphere of nitrogen, oxygen, or air.

*Observations on Location in Blood of Enzyme System Responsible for Pyruvate Removal*—Experiments were performed to determine which constituents of the blood contain the enzymes responsible for pyruvate removal. These experiments are summarized in Table IV. It was found that blood cells, obtained by washing whole blood three times with equal amounts of physiologic saline, removed considerably less pyruvate than equivalent amounts of the corresponding whole bloods. Plasma and heated blood cells (2 minutes at 100°) were completely inactive. The addition of heated plasma to unheated blood cells tended to restore the activity of the cells to that of whole blood. Heated cells to which unheated plasma had been added remained inactive.

The activity of the cells when suspended in phosphate buffer was considerably increased when glucose was added immediately before the addition of the pyruvate solution (Table V). Allowing the cells to stand for 1 hour at room temperature, in the presence of glucose, phosphate, and oxalate, before the addition of the pyruvate solution, increased their activity to that of oxalated whole blood incubated with phosphate (Table V). Addition of glucose to whole blood had no effect upon the rate of pyruvate removal.

*Observations on End-Product of Pyruvate Metabolism in Blood*—Measurements of the total bisulfite-binding substances and of the



pyruvate content of aliquot portions of the same bloods, after incubation with added pyruvate, showed that the amount of total carbonyl compounds which disappeared was equivalent to the

TABLE IV  
*Removal of Added Pyruvate by Blood Cells and Plasma*

	$Q_p^*$				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Whole blood.....	37	51	49	54	44
Cells.....	5	6	17	21	12
Plasma.....	0	1			0
Cells + plasma.....	39	49	52	56	45
Heated cells + plasma..	0	0		2	0
Cells + heated " ..	29	38	49	55	41

\*  $Q_p$  represents the micrograms of pyruvic acid removed by 1 ml. of blood or its equivalent of cells or plasma (hematocrit value) in 30 minutes, pyruvate being added 60 minutes after withdrawal of the blood from the vein.

TABLE V  
*Effect of Added Glucose on Pyruvate Removal by Blood Cells and Whole Blood*

Phosphate 0.075 M (pH 7.4); potassium oxalate, 0.1 per cent.

	Glucose added to 1 ml. blood	Period of incubation before sodium pyruvate addition	$Q_p$					
			Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
	mg.	min.						
Cells	0	0	23	14	26	41	28	22
	0	60	29	10	30	32	29	
	0.8	0	84	56	78	114	74	62
	0.8	60	143	137	115	159	150	134
Whole blood	0	60	150	130	121	161	147	141
	0.8	60	146	129	118	159	152	137

pyruvate removed. In fifteen experiments with and without added sodium fluoride (2 per cent) the average difference between the pyruvic acid (hydrazone method) and the total bisulfite-binding substances (expressed in micrograms of pyruvic acid) removed was  $\pm 3 \gamma$  (standard deviation  $\pm 1.8$ ) per ml. of blood.

Therefore, none of the pyruvate removed could have been converted into other carbonyl compounds.

The incubation of whole blood with added pyruvate, in the absence of fluoride, consistently resulted in the formation of more lactate than could be accounted for by the pyruvate removed (Table III). Incidentally, the glucose content of oxalated blood incubated with added pyruvate decreased to an appreciably greater extent than that of the same blood incubated for the same period without pyruvate (Table III).

TABLE VI

*Effect of Addition of 200  $\gamma$  of Sodium Pyruvate per Ml. of Blood on Lactate Formation in Oxalated, Fluorinized\* Blood, Incubated in 0.075 M Phosphate Buffer (pH 7.4)*

Experiment No.	Incubation with or without Na pyruvate for 30 min.	Pyruvic acid removed by 1 ml. blood	Lactic acid formed by 1 ml. blood	Glucose removed by 1 ml. blood
		$\gamma$	$\gamma$	$\gamma$
1	Without		0	-3
	With	66	59	15
2	Without		4	0
	With	88	73	8
3	Without		-2	5
	With	63	60	-20
4	Without		1	5
	With	99	90	0

\* Sodium fluoride (final concentration 2 per cent), pyruvate, and phosphate were added to oxalated blood after 60 minutes incubation at room temperature.

In Table VI are charted our results with blood to which 2 per cent fluoride had been added to prevent glycolysis. With this concentration of fluoride, the amount of lactate formed varied between 83 and 95 per cent of the pyruvate removed. Estimations of glucose content, before and after incubation, showed that little glycolysis had occurred. The inhibition of glycolysis is also indicated by the fact that no lactate formation occurred in the control bloods (with 2 per cent fluoride, without pyruvate) during the same period.

The results of simultaneous estimations of pyruvate removal and gas production (anaerobic) revealed that in one experiment de-

carboxylation accounted for 9 per cent of the pyruvate which disappeared, a value within the experimental error. In three other experiments no gas production occurred. There is then no indication that the anaerobic removal of pyruvic acid from blood is brought about through a dismutation yielding 50 per cent lactic acid and 50 per cent acetic acid plus  $\text{CO}_2$ , as has been reported for brain slices (15) and certain bacteria (16).

*Observations on Effect of Glycolysis on Pyruvate Removal*—As mentioned above, our first observations on the removal of pyruvate from heparinized and defibrinated bloods indicated a decreased activity of these bloods as compared with oxalated blood. More complete studies, which included measurements of lactate formation and glucose removal, showed the decrease in pyruvate removal to be associated with an increase in the amount of glycolysis (Table VII). When glycolysis was prevented by the addition of 2 per cent fluoride immediately upon withdrawal of the blood, the differences between the heparinized, defibrinated, and oxalated bloods were considerably less (Table III).

That the rate of the removal *in vitro* of pyruvate from heparinized blood is influenced, to a considerable extent, by the rate of glycolysis is demonstrated further by the fact that the addition of 2 per cent fluoride to heparinized blood, after glycolysis had been permitted to continue for 1 hour at room temperature, did not restore the pyruvate-removing powers of the blood to the level observed in an oxalated aliquot treated in a similar manner (Table I). The finding that the inhibiting effect of fluoride upon pyruvate removal was somewhat less striking with heparinized than with oxalated blood in these experiments (Table I) may be due to a greater counterbalancing effect of the inhibition of glycolysis by fluoride in heparinized blood.

Observations on the removal of pyruvate from heparinized blood in the absence of fluoride and of added pyruvate (Table VII) showed that the pyruvate content of such bloods steadily decreased during at least the first 30 minutes following withdrawal from the body. At the end of a 4 hour period of incubation, at room temperature, the pyruvate level had increased to above its original value. This latter phenomenon was not observed when oxalated blood was used (Table VII).

The results with oxalated and heparinized bloods to which 200

TABLE VII  
*Changes in Pyruvic Acid, Lactic Acid, and Glucose in Blood Allowed to Stand at Room Temperature*

	Precipitated after	Experiment 1		Experiment 2		Experiment 3		Experiment 4			Experiment 5		
		Pyruvic acid		Pyruvic acid		Pyruvic acid		Pyruvic acid		Glucose	Pyruvic acid		Glucose
		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	
Blood drawn in syringe containing iodoacetate (final concentration 0.5%) Blood drawn in dry syringe and allowed to stand in 25 mg. % heparin	2 min.	1.14	1.03	0.98									
	2 "	1.00	0.82	0.82									
	5 "	0.92	0.74	0.83									
	10 "	0.86	0.68	0.72									
	15 "	0.78	0.62	0.58									
	30 "	0.65											
	2 hrs.			0.75									
	4 "	1.34	1.10	1.11									
	Difference between pptn. at 3 min. and 4 hrs. after withdrawal from vein							+0.12	+20.7	-24.2	+0.13	+19.2	-23.3
	" "												
Blood allowed to stand in 0.2% potassium oxalate	" "							-0.31	+9.4	-13.4	-0.22	+8.0	-13.0
Blood allowed to stand in 0.5% sodium citrate	" "							+0.02	+18.7	-23.6	+0.39	+16.5	-27.8
Blood defibrinated	" "							0	+21.3	-20.4	+0.14	+20.2	-25.8

$\gamma$  of pyruvic acid per ml. of blood had been added are shown in Table III. While glycolysis in the heparinized blood was definitely inhibited by the addition of this amount of pyruvate, glucose removal and lactate formation were increased in the oxalated blood.

#### DISCUSSION

Our experiments with whole blood, plasma, and washed blood cells demonstrate that the enzyme (or enzymes) involved in the removal *in vitro* of pyruvate from blood is located within the blood cells. The adjuvant heat-stable factor present in plasma has been shown to be some intermediary metabolite of glucose rather than glucose itself. This interpretation is derived from the finding that the pyruvate-removing activity of blood cells is restored to that of whole blood by glucose only when a period of incubation intervenes between the addition of the glucose and the addition of the pyruvate, and also from the fact that whole oxalated blood, when permitted to stand for 30 to 60 minutes before addition of the pyruvate, removes more pyruvate than freshly drawn blood.

The activation of pyruvate disappearance by phosphate indicates a relationship between phosphorylation and glucose and pyruvate catabolism. This is also suggested by the finding that the addition of pyruvate to oxalated blood increased lactate formation to a greater extent in the presence of added phosphate than in its absence.

Embden and Deuticke (17) and Lohmann and Meyerhof (18) have shown that fluoride inhibits glycolysis at the phosphoglycerate stage. Our own experiments have shown that this inhibition is complete for the period of incubation (30 minutes) used in our studies, with a concentration of fluoride of 2 per cent. It would seem then that any lactate formed under these conditions, in blood containing 2 per cent fluoride, is derived from the pyruvate removed. As mentioned previously, the amount of lactate recovered in our experiments accounted for 83 to 95 per cent of the removed pyruvate.

We were unable to demonstrate conversion of pyruvate to other carbonyl compounds or to find any definite proof of pyruvate decarboxylation under our experimental conditions.

Dische (3) has reported that the removal of hexose diphosphate



by blood cells is increased by the addition of pyruvate. He found the hexose diphosphate to be converted into a substance which he considered to be phosphoglycerate. While Lu and Needham (19) did not find any lactate formation in defibrinated and fluorinized blood after adding pyruvate alone, they observed a significant lactate production when hexose diphosphate and adenylic acid were added together with the pyruvate. It is probable, then, that the phosphorylated metabolite of glucose which we have shown to catalyze the removal of pyruvate by washed blood cells is triose phosphate.

Our experiments indicate that the main pathway for pyruvate degradation in blood is identical with that described by Meyerhof and Kiessling (4) and Needham and Pillay (5) for muscle; namely, the coupling of pyruvate reduction with the oxidation of triose phosphate to phosphoglycerate. Needham and Pillay (5) found this reaction in muscle to be catalyzed by lactic acid dehydrogenase and coenzyme I (diphosphopyridine nucleotide). Iodoacetate, which inhibits the action of coenzyme I, also prevents the disappearance of pyruvic acid from the blood.

It is difficult to evaluate the statement of Flock, Bollman, and Mann (20) that, "The reduction of pyruvic acid to lactic acid does not occur in blood *in vitro*," as no mention is made of the anticoagulant used, or whether or not fluoride or phosphate was added. The increased removal of pyruvate from oxalated blood, as compared to heparinized or defibrinated blood, is associated with a partial inhibition of glycolysis, at the phosphoglycerate stage, by the oxalate. Thus there is first an inhibition of pyruvate and lactate formation from glucose catabolism, and second, an accumulation of triose phosphate and a corresponding stimulation of pyruvate removal.

In the heparinization and defibrination of blood no substance inhibiting glycolysis is added. Under these conditions pyruvate removal would tend to be counterbalanced by pyruvate formation. Further, as the amount of available triose phosphate decreases, the rate of pyruvate removal also decreases.

That glycolysis proceeds at a maximum rate in defibrinated and heparinized bloods is suggested by our observation that the addition of pyruvate to such bloods inhibited both glucose removal and lactate formation. The failure of Harris and Elgart (7) to demon-

strate a conversion to lactate of pyruvate added to blood cells is explicable, as in their experiments neither fluoride nor oxalate was used. Therefore the addition of pyruvate to the blood cells had an inhibiting effect on the lactate production from glucose, while in the control sample this reaction proceeded uninhibited (see Table III).

Our demonstration that there is a decrease in the pyruvate content of both heparinized and oxalated (2) blood beginning immediately following withdrawal of the specimen and continuing for at least 30 minutes is in disagreement with the statement of von Euler *et al.* (21). This finding indicates again the necessity for the immediate addition of iodoacetate to all blood used for the determination of its pyruvate content. It is therefore our custom, in such work, to withdraw the blood directly into a syringe containing the iodoacetate solution (22, 23).

#### SUMMARY

1. Defibrinated, heparinized, citrated, and oxalated human bloods remove pyruvate *in vitro*.

2. This removal begins immediately upon withdrawal of the blood and continues at an increasing rate for the first 30 minutes. In heparinized and defibrinated blood there is a subsequent increase in pyruvate content to above the original value, a phenomenon which has not been observed in oxalated blood.

3. Pyruvate removal by whole blood is increased by incubation with 0.075 M phosphate (optimum pH 7.4) and is unaffected by the addition of thiamine, cocarboxylase, and glucose or by the oxygen tension of the blood.

4. In the presence of 2 per cent fluoride, from 83 to 95 per cent of the pyruvic acid removed can be recovered as lactic acid. No evidence of decarboxylation or of conversion to other carbonyl compounds was found.

5. The enzyme system responsible for the removal of pyruvate by blood is heat-labile and is located within the blood cells. A heat-stable activator of this enzymatic reaction found in plasma appears to be a phosphorylated intermediary of carbohydrate catabolism, probably triose phosphate.

6. The evidence presented indicates an analogy between the mechanism for the anaerobic removal of pyruvate in blood and in muscle.

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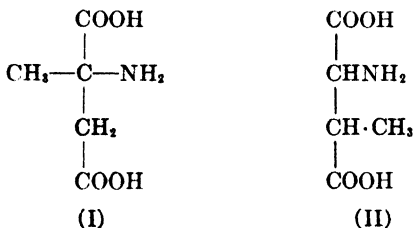
# ON METHYLASPARTIC ACIDS AND THEIR METHYLATION

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$\alpha$ -Methylaspartic acid (I) has long been known, having been obtained by Körner and Menozzi (1) by the hydrolysis of the products of heating citraconic, mesaconic, and itaconic esters with ammonia. Later on a more convenient synthesis from acetoacetic ester and ammonium cyanide was described by Zelinsky, Annenkoff, and Kulikoff (2). The isomeric acid,  $\beta$ -methylaspartic acid (II), which would seem to possess more biological possibilities appears not to have been described. The present communication deals with its synthesis and the products of methylation of both acids.

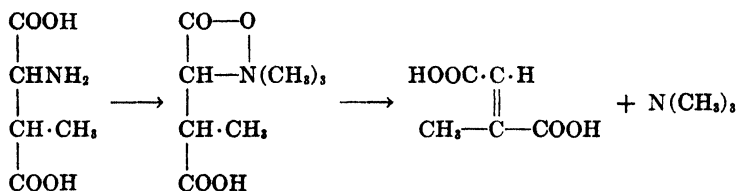


Both of the acids are isomeric with glutamic acid and might be designated as isoglutamic acids but, since the name isoglutamine has been adopted for the isomer of natural glutamine, such a designation might lead to confusion; hence the names  $\alpha$ - and  $\beta$ -methylaspartic acids seem preferable.  $\beta$ -Methylaspartic acid was prepared by a method analogous to that devised by Dunn, Smart, Redemann, and Brown (3) for the synthesis of glutamic acid. The sodium derivative of benzoylaminomalonic<sup>1</sup> diethyl

<sup>1</sup> Schmidt (4) states, "In 1931 Dunn and his coworkers described the synthesis of glutamic acid from  $\beta$ -chloropropionic ester and the *hitherto unknown benzoylaminomalonic ester*" (italics mine). As a matter of fact this benzoyl derivative was prepared and adequately characterized by Locquin and Cerchez (5) in 1928.

ester was condensed with  $\alpha$ -bromopropionic ethyl ester, and from the hydrolysis of the resulting product the desired amino acid was obtained in fair yield. No evidence was obtained of the presence of more than one of the two theoretically possible inactive isomers.  $\beta$ -Methylaspartic acid resembles aspartic acid in most respects but differs from both aspartic and  $\alpha$ -methylaspartic acids in yielding a copper salt which is extremely soluble in water.

The methylation of both  $\alpha$ - and  $\beta$ -methylaspartic acids with methyl sulfate was of interest for a variety of reasons. As is well known, aspartic acid is converted virtually quantitatively into fumaric acid on methylation and this reaction, in the writer's opinion, offers the best available method for the estimation and detection of aspartic acid. Both methylaspartic acids on methylation under mild conditions as regards temperature and alkalinity gave approximately 70 per cent of the theoretical yield of pure mesaconic acid free from isomers, together with tetramethylammonium sulfate. In addition each acid gave a base precipitated by phosphotungstic acid, which was unstable in the presence of excess of barium hydroxide used to decompose the precipitate. From this phosphotungstate precipitate an additional amount of mesaconic acid approaching 30 per cent of the theoretical amount was obtained in each case, together with trimethylamine. It would appear that the substances precipitated by phosphotungstic acid are the betaines of the respective methylaspartic acids and that they readily undergo typical decomposition with alkali.



The possibility that a methylaspartic acid might occur among the products of protein hydrolysis has often been considered. The foregoing methylation experiments appear to offer an opportunity to decide the question, since it should not be very difficult to detect significant amounts of mesaconic acid in admixture with the fumaric acid derived from natural aspartic acid. With

this end in view casein was hydrolyzed with dilute sulfuric acid and the acid removed with barium hydroxide in the usual fashion. On methylation of aliquot parts of the hydrolysate with excess of methyl sulfate and sodium hydroxide and subsequent extraction of the dibasic acids with ether, it was found that fumaric acid corresponding to 4.70 to 4.93 per cent of aspartic acid was obtained. The fumaric acid was weighed in the form of clean dry crystalline material and on fractional crystallization each crop, including the last minute amount from the mother liquors, melted at 287–288° in a closed capillary tube. It is concluded, therefore, that no mesaconic acid was present and that methylaspartic acids are not present among the products of casein hydrolysis.

In view of the fact that  $\beta$ -methylaspartic acid was found to yield a very soluble copper salt, a large amount of the crude dibasic acids from casein, obtained by twice precipitating the barium salts with alcohol, was converted into the copper salts and the soluble fraction separated. On removal of copper and subsequent methylation the only product in the ether extract was a little fumaric acid derived from aspartic acid.

The yield of fumaric acid obtained from hydrolyzed casein, as already stated, was equivalent to 4.70 to 4.93 per cent of aspartic acid. This result may be compared with 3.5 to 4.1 per cent of aspartic acid obtained by actual isolation of the acid as copper salt (6). In view of all the inherent difficulties attending isolation the two sets of figures appear reasonably concordant.

#### EXPERIMENTAL

*$\beta$ -Methylaspartic Acid*—Benzoylaminomalonic diethyl ester (42 gm.) was dissolved in 80 cc. of absolute alcohol. To this was added a cooled solution of 4.0 gm. of sodium dissolved in 75 cc. of alcohol. The sodium salt of the ester promptly separated out. An excess of ethyl  $\alpha$ -bromopropionate (40 gm.) was then added and the mixture boiled under a reflux for about 7 hours, when the reaction had become neutral. Alcohol was distilled off, water added, and the esters taken up in ether which was subsequently removed under diminished pressure. Hydrolysis was effected by 20 hours boiling with concentrated hydrochloric acid (150 cc.) and water (50 cc.). After cooling, benzoic acid was filtered off and the residual solution concentrated under diminished pressure. After addition of water

the evaporation was twice repeated to remove as much hydrochloric acid as possible. The residue was dissolved in 100 cc. of absolute alcohol, and to the clear solution aniline was added until Congo red paper was no longer turned blue. The crude acid that separated was filtered off and washed with alcohol and then recrystallized from boiling water in which it is only moderately soluble. The yield of acid was 10 gm. The substance crystallized in thick, hard, transparent hexagonal prisms and shows a marked tendency to form coherent crusts. It melts at 274–275° (uncorrected) with effervescence.

*Analysis*—Calculated. C 40.8, H 6.12, N 9.52

Found. " 40.8, " 6.26, " 9.54, amino N 9.60

50 mg. of the acid required 3.40 cc. of 0.1 N sodium hydroxide to give a faint pink color with phenolphthalein as indicator. This corresponds exactly with the calculated molecular weight of 147.

The copper salt was prepared by boiling an aqueous solution with excess of copper carbonate for 20 minutes. When filtered and slowly concentrated, no crystals separated, even on long standing. The salt is readily precipitated by alcohol, in which it is virtually insoluble, as a light blue powder which readily dissolves in water. Analysis showed it to be of normal composition (Cu = 29.9 per cent, calculated 30.5). When  $\beta$ -methylaspartic acid was heated with zinc dust, the vapors gave a strong pyrrole reaction but in the absence of zinc dust the reaction is practically negative.

*$\alpha$ -Methylaspartic Acid*—The acid was prepared by hydrolyzing with barium hydroxide solution the fractionated ester prepared from acetoacetic ester and ammonium cyanide according to Zelinsky's method. The properties of the substance agreed with those previously recorded.

*Methylation of  $\alpha$ - and  $\beta$ -Methylaspartic Acids*—Since the results were virtually identical in the case of the two acids, they may be considered together. Methylaspartic acid (1 gm.) was neutralized with 5 cc. of sodium hydroxide and then 6 cc. of methyl sulfate and 6 cc. of 33 per cent sodium hydroxide were added in eight divided portions in the course of 1 hour. The mixture was

allowed to stand for 2 hours, when the reaction had become feebly acid. Sulfuric acid was then added and the solution extracted with ether in a continuous extractor for 3 hours. On evaporation of the ether extract 0.55 gm. of pure mesaconic acid, melting at  $202^{\circ}$ , was obtained from the  $\alpha$  acid and 0.58 gm. from the  $\beta$  acid.

Calculated, C 46.2, H 4.62; found, C 46.1, H 4.66

On addition of concentrated perchloric acid (1 cc.) to the extracted fluids, 0.45 gm. of well crystalline tetramethylammonium perchlorate was obtained from the  $\alpha$  acid and 0.51 gm. from the  $\beta$  acid. The filtrates from the perchlorates were diluted and precipitated with phosphotungstic acid. The precipitate was dissolved in aqueous acetone, decomposed with excess of barium hydroxide, filtered, and at once acidified with hydrochloric acid. Barium was then quantitatively removed and the filtrate concentrated to small bulk and set aside to crystallize. From the  $\alpha$  acid 0.21 gm. of additional mesaconic acid, m.p.  $202^{\circ}$ , was obtained, while the  $\beta$  acid gave 0.31 gm. When platinic chloride was added to the filtrates from the second crops of mesaconic acid, the chloroplatinate of trimethylamine, decomposing at  $240-243^{\circ}$ , was obtained from each acid. Platinum 36.8, 36.7 per cent; calculated 36.9 per cent.

#### SUMMARY

The synthesis of  $\beta$ -methylasspartic acid is described. Both  $\alpha$ - and  $\beta$ -methylasspartic acids on methylation under prescribed conditions yield about 70 per cent of the theoretical amount of mesaconic acid, together with tetramethylammonium sulfate. The betaines of the two acids may be obtained on precipitation with phosphotungstic acid but on decomposition with barium hydroxide are decomposed with the formation of additional mesaconic acid, amounting to almost 30 per cent of the theoretical amount, and trimethylamine.

Hydrolyzed casein on methylation gives fumaric acid equivalent to 4.7 to 4.93 per cent of aspartic acid. No mesaconic acid could be detected and it is concluded that neither  $\alpha$ - nor  $\beta$ -methylasspartic acid is among the amino acids derived from casein.



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## STUDIES ON BIOLOGICAL OXIDATIONS

### XV. THE RATES OF REDUCTION OF THIAMINE AND DIPHOSPHOTHIAMINE

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Since the discovery of the chemical constitution of *cocarboxylase* (diphosphothiamine) by Lohmann and Schuster (1) numerous attempts have been made to explain the mechanism of diphosphothiamine action. The existence in its molecule of a quaternary nitrogen atom in a position comparable to its position in the diphosphopyridine nucleotides led some investigators to the belief that diphosphothiamine acts as a sluggish oxidation-reduction system (2). Weil-Malherbe (3) revived Langenbeck's hypothesis (4), and suggested that the first step of catalysis was the formation of a Schiff base followed by intramolecular oxidation-reduction. Weil-Malherbe's suggestion is not in agreement with the observations of Stern and Melnick (5). Recently Zinna and Williams (6) have suggested that thiamine is the reduced form of the active disulfide component; the fact that such a disulfide compound has 60 to 70 per cent activity (as compared with thiamine) in tests with pigeons is no proof of their contention, for the disulfide may be reduced in the animal tissues to thiamine and the thiamine phosphorylated, thus giving diphosphothiamine, the active component. The experiments reported in this paper on the rates of reduction of thiamine and diphosphothiamine are presented as evidence against the opinion that diphosphothiamine, as a component of enzyme systems, acts by being reversibly reduced and oxidized. These experiments have led us to postulate that the phosphorylated thiamine acts rather as the integral part of the activating protein and not as an electron acceptor.

## EXPERIMENTAL

The thiamine was generously provided by Merck and Company, Inc. Diphosphothiamine was prepared from thiamine, according to the method of Weijlard and Tauber (7). The purity of the preparation was established by nitrogen and phosphorus analysis. The sample used in these experiments had a nitrogen content of 11.5 per cent (theoretical, 11.71 per cent) and a phosphorus content of 12.4 per cent (theoretical, 12.96 per cent). The pyrimidine-pyridine compound (2-methyl-3-( $\alpha$ -hydroxyethyl)-N-(2-methyl-4-aminopyrimidyl-(5)-methyl)pyridine bromide hydrobromide) was kindly furnished by Dr. Franz C. Schmelkes. Fresh beer yeast was generously furnished by the Keeley Brewing Company of Chicago. Colloidal palladium was obtained from Farbenindustrie Aktiengesellschaft. Hydrogen and the gas mixtures of  $N_2:CO_2$  were freed from oxygen by being passed through a Pyrex tube 1 meter long filled with copper pellets and heated to  $500^\circ$ . The determination of gas uptake or output was made with the usual Warburg-Barcroft manometers.

*Rate of Reduction of Thiamine, Diphosphothiamine, and Pyrimidine-Pyridine with  $Na_2S_2O_4$* —Lipmann and Perlmann (8) have reported that for each molecule of thiamine reduced with  $Na_2S_2O_4$ , 2.75 molecules of  $CO_2$  were produced in bicarbonate buffer with  $N_2:CO_2$  as the gas phase, an indication that the reduction process involved only the quaternary nitrogen of the thiazole group. The reduction of thiamine, diphosphothiamine, and pyrimidine-pyridine was determined by the manometric method of Haas (9) in bicarbonate buffer, pH 8.0, with  $N_2:CO_2$  as the gas phase. With thiamine and diphosphothiamine, the reaction ended with an output of 590 c.mm. of  $CO_2$  per 0.01 mm of substance; i.e., 2.63 moles of  $CO_2$  per mole. With pyrimidine-pyridine, the reaction ended with an output of 616 c.mm., or 2.75 moles of  $CO_2$  per mole. The reduction of pyrimidine-pyridine by  $Na_2S_2O_4$ , like that of thiamine (pyrimidine-thiazole), seems to affect only the pyridine half of the molecule. Whether the reduction of pyrimidine-pyridine by  $Na_2S_2O_4$  is followed by the separation of the molecule into its component parts (pyrimidine and pyridine), as happens in the reduction of thiamine (10), is not known.

The rate of reduction of thiamine was more than 3 times that of diphosphothiamine, for in the first case half reduction was reached

in 4 minutes, while in the second it required 15 minutes. Half reduction of pyrimidine-pyridine required 25 minutes (Table I). It has been reported by Schmelkes (11) and by Baumgarten and Dornow (12) that this compound has some vitamin B<sub>1</sub> activity in tests with rats and pigeons.

*Reduction with Platinum Black and Colloidal Palladium*—There are conflicting reports on the extent of reduction of thiamine by hydrogen with platinum or palladium as catalyst. According to Kuhn and Vetter (13), 1 mole of thiamine in 0.1 M HCl took up 1.08 moles of H<sub>2</sub> with palladium oxide as catalyst. Lipmann and

TABLE I

*Reduction of Thiamine, Diphosphothiamine, and Pyrimidine-Pyridine with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, Pt Black, and Colloidal Pd; Comparative Rates of Reduction*

Reduction with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> was carried out at pH 8.0; with Pt or Pd, at pH 7.4. Temperature, 38°; amount of substance, 0.01 mm.

Reductant	Thiamine		Diphospho- thiamine		Pyrimidine- pyridine	
	CO <sub>2</sub> output or H <sub>2</sub> uptake per mole	Time of half reduc- tion	CO <sub>2</sub> output or H <sub>2</sub> uptake per mole	Time of half reduc- tion	CO <sub>2</sub> output or H <sub>2</sub> uptake per mole	Time of half reduc- tion
	<i>moles</i>	<i>min.</i>	<i>moles</i>	<i>min.</i>	<i>moles</i>	<i>min.</i>
Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	2.63	4	2.63	15	2.80	25
Pt black, 100 mg.	3.60	16	2.84	42	5.60	20
Colloidal Pd, 4 mg.	1.32	14	1.55	45	2.59	23
“ “ 20 “	2.90		2.60			

Perlmann reported at pH 8.0 an uptake of 0.92 mole of H<sub>2</sub> and at pH 10.5 an uptake of 1.07 moles. According to Stern and Melnick, the amount of hydrogen taken up was not constant. Our observations are in agreement with those of Stern and Melnick, the amount of H<sub>2</sub> taken up depending on the amount of catalyst used. With large amounts of platinum black or colloidal palladium, there was, in the reduction of 1 mole of thiamine by platinum, an uptake of 3.6 moles of hydrogen and in the reduction by colloidal palladium an uptake of 1.32 moles of hydrogen. In the reduction of diphosphothiamine by platinum black there was an uptake of 2.84 moles of H<sub>2</sub> and by colloidal palladium an uptake

of 1.55 moles of  $H_2$  (Table I). With smaller amounts of catalyst the hydrogen uptake stopped before these figures were reached, as in the experiments of Stern and Melnick.

This lack of consistency in the extent of reduction of thiamine and diphosphothiamine may be due to the formation of a compound between platinum and the sulfur group of thiamine with concomitant loss or impairment of catalytic power. In fact, the catalytic decomposition of  $H_2O_2$  by bright platinum was com-

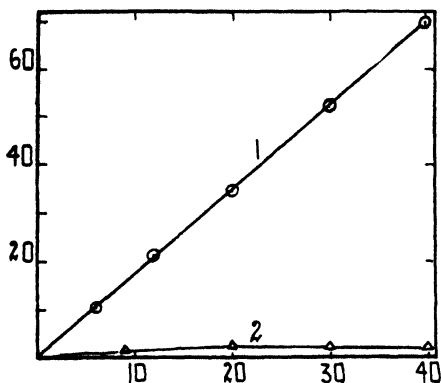


FIG. 1

FIG. 1.  $H_2O_2$  decomposition by bright platinum. pH 7.4; temperature  $28^\circ$ . Inhibiting effect of diphosphothiamine. Curve 1, control; Curve 2, diphosphothiamine (0.001 M). Abscissa, time in minutes; ordinate,  $O_2$  output in c.mm.

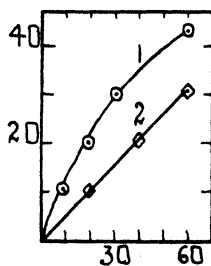


FIG. 2

FIG. 2. Oxidation of reduced thiamine and diphosphothiamine with histidine-ferriprotoporphyrin. Abscissa, time in minutes; ordinate,  $O_2$  uptake in c.mm., pH 7.4; temperature  $38^\circ$ . Curve 1, thiamine; Curve 2, diphosphothiamine.

pletely inhibited by 0.001 M diphosphothiamine (Fig. 1). The substitution of the thiazole group of vitamin  $B_1$  for the pyridine group, as in pyrimidine-pyridine, made the compound more easily affected by colloidal palladium and platinum black. On reduction of pyrimidine-pyridine by colloidal palladium (4 mg.) the uptake of hydrogen stopped when 2.59 moles were taken up per mole of pyrimidine-pyridine; with platinum black reduction of the compound was more extensive, for there was an uptake of 5.6 moles of hydrogen per mole.

As in the reduction with  $\text{Na}_2\text{S}_2\text{O}_4$ , the rate of reduction of thiamine with either Pt black or colloidal Pd was about 3 times as great as that of diphosphothiamine. 0.015 mm of thiamine reduced with colloidal Pd and  $\text{H}_2$  took up 0.5 mole of  $\text{H}_2$  in 16 minutes, while diphosphothiamine took up an equivalent amount of  $\text{H}_2$  in 42 minutes. Under similar conditions, pyrimidine-pyridine required 23 minutes with colloidal Pd, and 20 minutes with Pt black (Table I).

*Reoxidation of Reduced Thiamine and Diphosphothiamine*—Reduced thiamine and diphosphothiamine are not oxidized by atmospheric oxygen. They were slowly oxidized (pH 7.63) by histidine-ferriprotoporphyrin. Whether reduced by  $\text{Na}_2\text{S}_2\text{O}_4$ , by platinum, or by palladium, the rate of reoxidation of diphosphothiamine was lower than that of thiamine (Fig. 2). Neither reduced thiamine nor diphosphothiamine was oxidized by the alloxazine dinucleotide prepared according to Warburg and Christian (14) and active in the oxidation of pyruvate by Lipmann's acetone preparation from *Bacillus delbrückii*.

Neither reduced diphosphothiamine nor the reoxidized compound (reoxidized with histidine-ferriprotoporphyrin) had cocarboxylase activity when added to alkaline washed yeast or coenzyme activity for the oxidation of pyruvate by the acetone powder of *Bacillus delbrückii*. (No data on these experiments are given, because the results are in agreement with those published by Stern and Melnick (15).)

Numerous attempts to reduce thiamine and diphosphothiamine at pH 7.4 with reduced methyl viologen (with an  $E'_0$  close to that of hydrogen (16)) and with rosinduline GG ( $E'_0$   $-0.305$  volt (17)) were unsuccessful. Chromous acetate also failed to reduce either compound.

All these experiments show that thiamine becomes more resistant to the action of oxidizing and reducing agents after phosphorylation, and are an indication that the action of diphosphothiamine as a component of enzyme systems is not the result of reversible oxidation and reduction.

#### SUMMARY

Thiamine becomes on phosphorylation to diphosphothiamine more resistant to the action of oxidizing and reducing agents; thus

the rate of reduction of thiamine with  $\text{Na}_2\text{S}_2\text{O}_4$ , colloidal palladium, and platinum black is about 3 times as fast as the rate of reduction of diphosphothiamine. The rate of reoxidation of reduced thiamine is also faster than that of diphosphothiamine.

Neither reduced diphosphothiamine nor reduced diphosphothiamine reoxidized by histidine-ferriprotoporphyrin showed activity as the vitamin or enzyme component.

It is concluded from these experiments that diphosphothiamine acts as a component of enzyme systems by forming an integral part of the activating protein.

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## STUDIES ON BIOLOGICAL OXIDATIONS

### XVI. THE EFFECT OF THIAMINE ON CONDENSATION REACTIONS OF PYRUVATE

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In Paper XV (1) it has been shown that thiamine becomes on phosphorylation to diphosphothiamine more resistant to the action of oxidizing and reducing agents, a fact which is contrary to the opinion that phosphorylated thiamine acts, in its enzymatic activity, as a sluggish reversible oxidation-reduction system. An explanation of the functions of diphosphothiamine must, therefore, take into consideration those experiments. The fact that diphosphothiamine is a component of the enzyme systems which decarboxylate  $\alpha$ -ketocarboxylic acids, which oxidize pyruvate, and which dismute pyruvate must also be considered. Such multiple catalytic functions suggest the possibility that diphosphothiamine acts by forming an integral part of the activating protein of the enzyme systems concerned with the activation of pyruvate. Once pyruvate is activated, it may react with catalysts for its oxidation, dismutation, decarboxylation, carboxylation, or condensation. This hypothesis need not postulate reversible oxidation-reduction; it predicts, on the other hand, more catalytic functions for the vitamin than those hitherto known, a prediction quite in harmony with the profound metabolic disorders found in animals suffering from thiamine deficiency. The validity of this hypothesis was tested in experiments with the tissues of thiamine-deficient rats and chickens by observing the effect of thiamine on the biologically important reactions in which pyruvate is one of the reactants; namely, the *condensation reactions*. In this paper experiments are presented which show that thiamine, probably as diphospho-



thiamine, accelerated the following condensation reactions, the synthesis of carbohydrate, of  $\alpha$ -ketoglutarate, of citrate, of acetoacetate, and of succinate. Furthermore, experiments are presented showing for the first time that thiamine accelerates the oxygen consumption of skeletal muscle of avitaminotic rats in the presence of pyruvate. The rôle of thiamine in cellular metabolism becomes thus one of primary importance.

#### EXPERIMENTAL

For the experiments with animal tissues, young rats reared at the laboratory and young white Leghorn chickens were used. The rats were fed the diet recommended by Ammerman and Waterman (2), the casein in this diet being vitamin-free casein instead of technical casein. The chickens were fed the diet of Elvehjem (3). The control animals were fed the same diets plus thiamine. In experiments in which chemical analyses were made, the excised tissues (sliced or ground) were placed in special wide mouthed Pyrex flasks which communicated with each other through glass tubing, the two ends being provided with stop-cocks through which rapid saturation with the chosen gas mixtures was effected. These batteries of vessels were shaken at 38°. The blood "pyruvate" was determined colorimetrically with the Pulfrich photometer by the method of Lu (4); pyruvate in the tissue suspensions was determined manometrically by a modification of the carboxylase method which will be described in a later paper. Succinate was determined manometrically by measuring oxygen consumption during oxidation to fumarate with thoroughly washed pigeon muscle suspensions in the presence of cresyl blue as oxidizing catalyst. Fine suspensions of muscle were prepared by chopping small pieces of breast muscle for 10 minutes in a Waring electric mixer with 150 cc. of water and an equal volume of ice. The suspension was centrifuged for 30 minutes in a room at 3°, the supernatant fluid was poured off, and the paste poured into the mixer, where it was washed once more with ice and water. After 30 minutes centrifugation, the supernatant fluid was poured off and the paste suspended in 3 volumes of 0.03 M phosphate, pH 7.4. 0.8 cc. of this suspension was used for each determination.  $\alpha$ -Ketoglutarate was determined as succinate after its oxidation with permanganate (5). Citrate was determined colorimetrically by the method of

Pucher *et al.* (6). Alanine was determined as lactic acid by a modification of Kendall and Friedemann's method (7) which will be described in a separate communication. Acetoacetate was determined manometrically by decarboxylation with aniline citrate at 38° (8). Carbohydrates were determined according to the procedure used by Lyman and Barron (9). The tissue proteins were precipitated with either metaphosphoric acid, trichloroacetic acid, or tungstate, depending upon the analysis to be made. In experiments with tissue slices the buffers were Ringer-phosphate or Ringer-bicarbonate, the Ringer's solution being modified from that of Hastings *et al.* (10). (The concentration of phosphate buffer in the Ringer's solution was 0.01 M, instead of 0.005 M as used by Hastings.) With tissue chopped or minced in a Latapie grinder, the Ringer's solution contained no calcium and the NaCl was 0.1 M.

Numerous failures during the first months of this work were due to the use of animals in different stages of deficiency. Only with animals in the last stages of deficiency was the effect of added thiamine in *in vitro* experiments consistent, the blood "pyruvate" then being from 6 to 13 mg. per cent. Another source of failure was the addition of substrate and thiamine simultaneously. In order to become an enzyme component, thiamine must first be phosphorylated. In all the experiments reported in this paper the tissues were incubated with thiamine (50  $\gamma$ ) for 30 minutes previous to the addition of substrates.

*Synthesis of Carbohydrate*—The most important condensation reaction of pyruvate is that which ends in the synthesis of carbohydrate. The rat kidney was chosen to test the effect of thiamine on this synthesis because Benoy and Elliott (11) had previously found that rat kidney slices incubated in Ringer-bicarbonate with  $O_2:CO_2$  as the gas phase converted pyruvate into carbohydrate determined as glucose. These experiments were performed with litter mate rats, half of which were kept on a thiamine-free diet, the other half on the same diet plus thiamine. Kidney slices of normal rats synthesized 5.52 micromoles of carbohydrate per gm. of fresh tissue on addition of pyruvate, and 6.25 micromoles on addition of pyruvate plus fumarate. Kidney slices of avitaminotic rats synthesized 2.97 micromoles on addition of pyruvate, and 2.77 micromoles on addition of pyruvate plus fumarate, a decrease of 45

and 56 per cent, respectively, from the rates of synthesis found in normal rats. The addition of thiamine (50  $\gamma$ ) to kidney slices of avitaminotic rats restored to normal the rate of carbohydrate synthesis (Table I). (The figures given in all these experiments represent carbohydrate found at the end of the experiment minus the initial carbohydrate.)

While the rôle of thiamine in the synthesis of carbohydrate is thus demonstrated, the exact mechanism of the thiamine action is

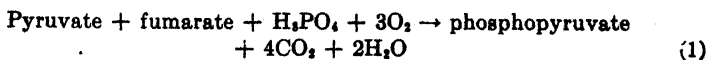
TABLE I

*Effect of Thiamine on Synthesis of Carbohydrate by Kidney Slices (Rat)*

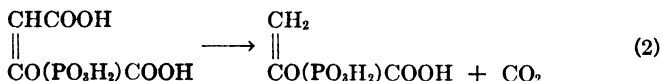
Buffer, Ringer-bicarbonate; gas phase,  $O_2:CO_2$ ; pH 7.4; pyruvate concentration, 0.02 M; fumarate, 0.01 M; thiamine (vitamin  $B_1$ ), 50  $\gamma$ ; incubation time, 3 hours at 38°.

State of rat	Carbohydrate formation, micromoles glucose per gm. tissue			
	No substrate	Pyruvate	Pyruvate + fumarate	Pyruvate + fumarate + $B_1$
Normal	1.0	5.60	7.00	7.16
"	1.11	6.07	6.90	6.68
"	1.39	4.89	4.85	4.89
Thiamine-deficient	1.55	3.27	2.50	5.06
"	2.11	4.33	4.05	7.66
"	1.55	2.83	3.61	9.70
"	0.89		2.00	5.11
"	0.94	1.45	1.73	5.40
Average normal	1.17	5.52	6.25	6.24
" thiamine-deficient	1.41	2.97	2.77	6.59

still unsolved. Under the conditions of these experiments (tissue slices in Ringer-bicarbonate) synthesis did not take place in the absence of oxygen, nor in the presence of fluoride. Addition of fumarate in a ratio of 10 pyruvate to 1 fumarate increased the rate of synthesis slightly (11 per cent), further increase of fumarate concentration having no effect (Table II). On the basis of these experiments Barron and Lyman (12) postulated that synthesis of carbohydrate might start with an oxidative condensation of pyruvate and fumarate to give phosphopyruvate.



Thiamine would catalyze this condensation. However, numerous experiments in which kidney (chopped and in slices) and muscle (chopped skeletal muscle) were incubated with pyruvate in the presence of fluoride failed to show phosphopyruvate formation. If experimental confirmation is obtained for Lipmann's interesting suggestion (13) that phosphopyruvate may be formed by decarboxylation of phosphooxalacetic acid,



the effect of thiamine may be exerted either in the synthesis of phosphooxalacetic acid (carboxylation) or in its decarboxylation.

TABLE II

*Effect of Fumarate on Synthesis of Carbohydrate and Citrate from Pyruvate by Kidney Slices (Rat)*

Carbohydrate synthesis took place in 3 hours; citrate synthesis, in 30 minutes. The figures represent micromoles per gm. of fresh tissue.

Substrates	Glucose	Citrate
None.....	1.11	0
Pyruvate (0.02 M).....	3.78	0.14
“ + fumarate (0.002 M).....	4.18	2.64
“ + “ (0.01 “).....	3.73	3.05
“ + “ (0.02 “).....	3.05	2.48

Once phosphopyruvate is formed, the synthesis of carbohydrate proceeds through the same steps as those found in its degradation, since all the remaining reactions are reversible.

*Synthesis of  $\alpha$ -Ketoglutarate*—To test the effect of thiamine on the synthesis of  $\alpha$ -ketoglutarate from pyruvate, the liver was chosen, because this tissue readily produces  $\alpha$ -ketoglutarate, as demonstrated by Evans (14). Addition of thiamine accelerated the synthesis of  $\alpha$ -ketoglutarate by liver slices of avitaminotic animals (rats and chickens). In Ringer-phosphate with  $\text{O}_2$  as the gas phase, in the presence of 0.04 M pyruvate and *l*-malate, and after incubation at  $38^\circ$  for 30 minutes, there was an average of 2.65 micromoles of  $\alpha$ -ketoglutarate formed by the liver of avitaminotic rats, and 0.87 micromole by the liver of avitaminotic chickens. In the presence of thiamine these figures rose to 3.17

and 1.36 micromoles, respectively (Table III). Since in these experiments the malate concentration was equivalent to that of pyruvate, the thiamine effect was not due to increased carboxylation of pyruvate to oxalacetate. The mechanism of  $\alpha$ -ketoglutarate formation is still unknown. However, any hypothesis

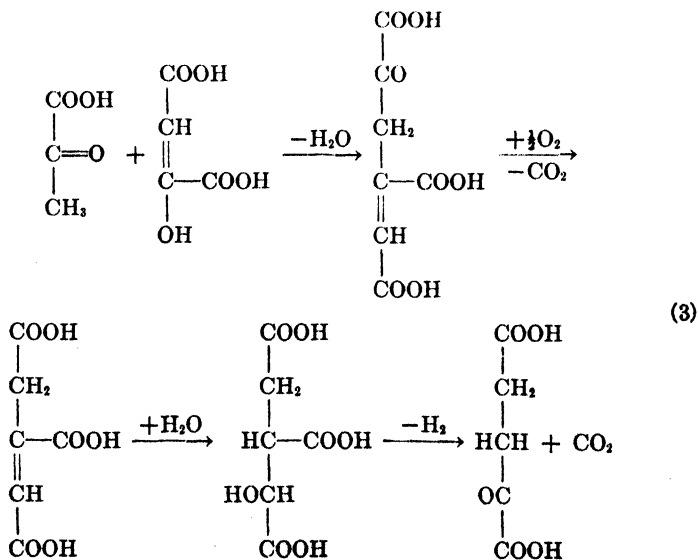
TABLE III

*Effect of Thiamine on Synthesis of  $\alpha$ -Ketoglutarate from Pyruvate by Liver of Thiamine-Deficient Animals*

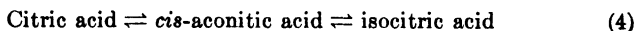
Concentration of pyruvate and *l*-malate, 0.04 M; thiamine (vitamin B<sub>1</sub>), 50  $\gamma$ ; incubation time, 30 minutes.

Liver (vitamin B <sub>1</sub> -deficient)	Ketoglutarate synthesis, micromoles per gm. tissue	
	Pyruvate + malate	Pyruvate + malate + B <sub>1</sub>
Rat.....	3.50	3.92
".....	2.37	2.95
".....	2.08	2.63
Chicken.....	0.87	1.36

explaining the mechanism must agree with two recent observations, (1) that CO<sub>2</sub> takes part in the synthesis (15), (2) that citric acid is



not an intermediate in the synthesis (16). To avoid the citric acid steps, Wood *et al.* (16) go back to one of the schemes postulated by Krebs (17), according to which the condensation of pyruvate and enoloxalacetic acid would give oxalomesaconic acid, which by oxidative decarboxylation would form *cis*-aconitic acid (Reaction 3). Aconitase seems to form two different isomeric compounds, citric and isocitric acids, through addition of water to a double bond.



At equilibrium there is 16 per cent of isocitric acid, 4 per cent of *cis*-aconitic acid, and 80 per cent of citric acid (18, 19). The synthesis of  $\alpha$ -ketoglutarate would depend then on the rate of condensation of pyruvate and oxalacetate, and on the value of the equilibrium constants for the reactions catalyzed by aconitase, for a deviation of the equilibrium towards the left side of the reversible reactions (citrate formation) would result in a diminished  $\alpha$ -ketoglutarate formation.

*Synthesis of Citrate*—In Ringer-phosphate, aerobically, chopped heart of normal rats produced 0.14 micromole of citrate in 30 minutes in the presence of 0.02 M pyruvate; on addition of 0.002 M fumarate it produced 19 times as much, 2.64 micromoles. There was a further increase in citrate formation when the ratio of pyruvate to fumarate became 2:1. It diminished when the ratio was 1:1 (Table II). To test the effect of thiamine on the formation of citrate from pyruvate, the latter ratio of pyruvate to fumarate was used. In Table IV are given the results of experiments performed with litter mate rats. The chopped heart of normal rats produced about 1.65 micromoles of citrate per gm. of tissue, and 1.68 micromoles on addition of thiamine. The heart of thiamine-deficient rats produced 0.67 micromole of citrate, a decrease of 57 per cent from the normal rate under the conditions of the experiment, and 0.71 micromole on addition of thiamine. Chopped brain of normal rats produced 0.407 micromole of citrate and that of deficient rats 0.369 micromole, addition of thiamine increasing these amounts very little (0.416 micromole with normal rats and 0.386 with deficient rats). Kidney slices of thiamine-deficient rats formed 0.313 micromole of citrate, and on addition of thiamine 0.424 micromole, an increase of 35 per cent. The rôle of thiamine

in the synthesis of citrate has also been demonstrated in experiments *in vivo* with rats by Sober, Lipton, and Elvehjem (20). The

TABLE IV

*Effect of Thiamine on Synthesis of Citrate by Rat Tissues*

Buffer, Ringer-phosphate; O<sub>2</sub> as gas phase; pH 7.4; concentration of pyruvate, and of fumarate or malate, 0.0446 M; thiamine (vitamin B<sub>1</sub>), 50 γ; incubation time, 30 minutes.

Tissue	Citrate synthesis, micromoles per gm. tissue	
	Pyruvate + malate	Pyruvate + malate + B <sub>1</sub>
Chopped heart, normal	1.38	1.44
“ “ “	2.28	2.12
“ “ “	1.56	1.61
“ “ “	1.37	1.56
“ “ deficient	0.68	0.85
“ “ “	1.11	1.19
“ “ “	0.68	0.57
“ “ “	0.40	0.45
“ “ “	0.47	0.486
Kidney slices, “	0.29	0.365
“ “ “	0.24	0.375
“ “ “	0.448	0.60
“ “ “	0.276	0.36
“ “ “	0.310	0.42
Chopped brain, normal	0.395	0.448
“ “ “	0.550	0.563
“ “ “	0.485	0.440
“ “ “	0.200	0.214
“ “ deficient	0.571	0.570
“ “ “	0.336	0.385
“ “ “	0.280	0.297
“ “ “	0.287	0.292
Averages		
Heart, normal	1.65	1.68
“ deficient	0.67	0.71
Kidney, “	0.313	0.424
Brain, normal	0.407	0.416
“ deficient	0.369	0.386

urinary excretion of citric acid, somewhat lower in polyneuritic rats, rose after the administration of thiamine.

*Synthesis of Succinate*—The formation of succinate from pyru-

vate by the kidney was demonstrated by Elliott and Greig (21). The effect of thiamine on the synthesis of succinate by kidney slices of avitaminotic rats was studied by inhibiting the oxidation of succinate with 0.02 M malonate. Kidney slices were incubated aerobically in Ringer-phosphate containing 0.02 M pyruvate, fumarate, and malonate (Table V). Under these conditions there was in the presence of thiamine an increase in the oxygen uptake, in the utilization of pyruvate, and in the formation of succinate.

*Synthesis of Acetoacetate*—To test the effect of thiamine on the synthesis of acetoacetate from pyruvate, the liver was chosen. A great species difference in the rate of acetoacetate formation was

TABLE V

*Effect of Thiamine on Utilization of Pyruvate and on Synthesis of Succinate by Kidney of Thiamine-Deficient Rats*

Buffer, Ringer-phosphate; the buffer contained 0.02 M pyruvate, 0.02 M fumarate, and 0.02 M malonate. The figures represent c.mm. per 100 mg. of wet weight.

Experiment No.....	I	II	III	IV
Time of incubation, min.....	120	180	120	180
O <sub>2</sub> uptake, control.....	527	901	623	818
“ “ “ + 50 γ thiamine.....	644	1071	726	992
Pyruvate used, control.....	?	492	546	422
“ “ “ + 50 γ thiamine.....	?	626	805	501
Succinate formed, control.....	65.4	138	151	?
“ “ “ + 50 γ thiamine.....	96.0	186	174	?

found, avian liver producing large amounts while rat liver produced very little. Furthermore, while acetoacetate formation was readily observed in ground avian liver, rat liver produced none when subjected to similar treatment (Table VI). The experiments with rat liver were consequently performed with slices, while those with avian liver were performed with ground tissue. Avian liver, suspended in ice-cold 0.9 per cent NaCl, was crushed in the homogenizer of Potter and Elvehjem (22). The suspension was centrifuged at 3000 R.P.M. for 5 minutes, the supernatant fluid was discarded, and the precipitate resuspended in 50 volumes of ice-cold NaCl solution and centrifuged again. The precipitate was resuspended in ice-cold 0.05 M sodium phosphate buffer, pH 7.4 (2 volumes of buffer per gm. of fresh liver). 1 cc. of this suspension



was added to the Warburg vessels, which already contained 0.6 cc. of 0.4 M NaCl, 0.1 cc. of a mixture of  $MgCl_2$  and  $MnSO_4$  (0.01 M), 0.2 cc. of lithium pyruvate to make the solution 0.007 M, and water to 2 cc. Since suspensions of this kind do not phosphorylate thiamine, 50  $\gamma$  of diphosphothiamine were added for the vitamin effect. Acetoacetate formation by the liver of avitaminotic animals (chicken and rat) was distinctly low as compared with its formation by the liver of normal animals; while the liver of control chickens produced 0.36 micromole of acetoacetate per 100 mg. of

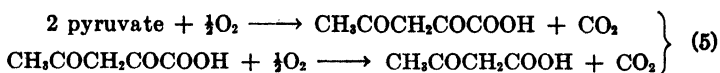
TABLE VI

*Effect of Thiamine on Synthesis of Acetoacetate from Pyruvate by Liver*

Pyruvate concentration, 0.007 M; amount of thiamine (vitamin  $B_1$ ), 50  $\gamma$ ; incubation time, 90 minutes. The figures represent micromoles per 100 mg. of fresh tissue for the liver of the chicken, per mg. of dry weight for the liver of the rat.

Tissue	O <sub>2</sub> uptake			Acetoacetate formation	
	Control	Pyruvate	Pyruvate + $B_1$	No thiamine	Thiamine
Pigeon liver, ground, normal.....	0.143	0.98		0.470	
Chicken liver, ground					
Normal.....	0.058	1.17		0.36	
$B_1$ -deficient.....	0.056	0.335	0.465	0.079	0.134
".....	0.046	0.085	0.160	0.029	0.062
".....	0.089	0.152	0.201	0.032	0.047
".....	0.053	0.06	0.070	0.023	0.036
Rat liver, slices					
Normal.....	0.062	0.077		0.005	
" + $NH_4Cl$ , 0.04 M.....		0.067		0.025	
$B_1$ -deficient + $NH_4Cl$ .....	0.049	0.050	0.057	0.0083	0.0085

fresh tissue in 90 minutes, the liver of avitaminotic chickens produced an average of 0.04 micromole, only one-tenth as much. On addition of diphosphothiamine, the acetoacetate formation (average) was 0.07 micromole, an increase of 73 per cent; there was also an increase of 41 per cent in the oxygen consumption. Krebs and Eggleston (23) have also reported an increased acetoacetate synthesis by the avitaminotic pigeon liver on addition of thiamine. The synthesis of acetoacetate from pyruvate seems to require the condensation of 2 molecules of pyruvate to acetopyruvate and the oxidative decarboxylation of acetopyruvate to acetoacetate (24).



Thiamine may act by catalyzing either the condensation reaction or the oxidative decarboxylation of acetoxyruvate.

*Effect of Thiamine on Transamination by Liver of Avitaminotic Rats*—Braunstein and his coworkers (25) and Cohen (26) have studied extensively the reversible reaction between pyruvate and *l*(+)-glutamate with formation of alanine and  $\alpha$ -ketoglutarate.



The reaction is catalyzed by transaminase, an enzyme system of as yet unknown components. Experiments performed with the liver of avitaminotic rats have shown that thiamine is not a component of transaminase. In these experiments chopped liver was incubated, with  $\text{N}_2$  as the gas phase, in the presence of pyruvate and *l*(+)-glutamate (0.03 M). At the end of 40 minutes, the proteins were precipitated, and alanine was deaminated to lactate, which was extracted with ether for 3 hours and determined with the method of Friedemann and Graeser (27). The liver of avitaminotic rats produced 0.228 micromole of alanine per gm. of liver; on addition of thiamine it produced 0.230 micromole. However, in one experiment there was an increase of 34 per cent on addition of thiamine. One objection to these experiments is that the time of incubation was too long (40 minutes), for Cohen (26) has recently shown that equilibrium is reached very quickly.

*Thiamine As Catalyst for Carboxylation of Pyruvate*—It has been postulated by Krebs and Eggleston (23) that the only rôle of thiamine is that of catalyst for the reaction of Wood and Werkman; i.e., the carboxylation of pyruvate to oxalacetate. Condensation reactions of pyruvate ought to proceed, therefore, at the same rate in avitaminotic tissues when both pyruvate and oxalacetate are added. However, the accelerating effect of thiamine on the numerous condensation reactions reported here was obtained in the presence of pyruvate and oxalacetate (fumarate and malate undergo rapid change to oxalacetate). The experiments reported in Table VII provide further evidence that the rôle of thiamine is more complex than that of catalyst for the carboxylation of

pyruvate. Kidney slices of avitaminotic rats were incubated for 150 minutes in the presence of (1) pyruvate, (2) pyruvate plus thiamine, (3) pyruvate plus *l*-malate, and (4) pyruvate plus malate plus thiamine. According to the theory of Krebs, the utilization of pyruvate in the presence of malate ought to be independent of the presence of thiamine, since the product of thiamine catalysis, oxalacetate, was already supplied. In the presence of pyruvate and malate, the kidney of avitaminotic rats utilized 0.034 micromole of pyruvate per mg. of dry weight; addition of thiamine raised the utilization to 0.223 micromole, a 7-fold increase. Krebs' theory, although attractive for its simplicity, is untenable in the light of all the experiments reported here. Thiamine may act

TABLE VII

*Effect of Thiamine on Utilization of Pyruvate in Presence of l(-)-Malate. Kidney Slices of Thiamine-Deficient Rat*

Buffer, Ringer-NaHCO<sub>3</sub>; gas phase, O<sub>2</sub>:CO<sub>2</sub>; pH 7.4; concentration of pyruvate and *l*(-)-malate, 0.005 M; thiamine (vitamin B<sub>1</sub>), 50  $\gamma$ ; incubation time, 150 minutes.

Substrates	Pyruvate utilization, micromoles per mg. dry weight
Pyruvate .....	0.172
" + B <sub>1</sub> .....	0.303
" + malate .....	0.034
" + " + B <sub>1</sub> .....	0.223

as a catalyst for the Wood and Werkman reaction, but there is as yet no experimental evidence for this contention.

*Effect of Thiamine on Respiration of Muscle and Utilization of Pyruvate*—The increase in the oxygen consumption and pyruvate utilization of avitaminotic tissues on addition of thiamine has been demonstrated by a number of investigators in the heart muscle, brain, kidney, and liver, but all experiments performed with diaphragm or skeletal muscle have been reported as giving negative results; *i.e.*, no increase on thiamine addition. With strips of diaphragm from avitaminotic rats in prolonged experiments (5 hours) it was possible to demonstrate the effect of thiamine on muscle, not only on the oxygen consumption but also on the utilization of pyruvate. In the experiments reported in Table VIII the oxygen consumption was increased significantly only after

the 3rd hour of incubation, the increase being observed in other experiments at the end of the 2nd hour. The increased respiration was accompanied by an increase in the utilization of pyruvate. Similar results were obtained with strips of abdominal muscle. The absence of thiamine effect during the 1st hour must be attributed to slow penetration and phosphorylation of thiamine, for it is only as diphosphothiamine that the vitamin is active as the enzyme component. The failure of other investigators to detect the vitamin effect in muscle was probably due to either the use of thick strips of diaphragm, injury, or short time experiments.<sup>1</sup>

TABLE VIII

*Effect of Thiamine on O<sub>2</sub> Uptake and Pyruvate Utilization by Diaphragm of Thiamine-Deficient Rats*

Buffer, Ringer-phosphate, pH 7.4; O<sub>2</sub> as gas phase; temperature 38°; pyruvate concentration, 0.007 M; thiamine, 50  $\gamma$ .

Hour	O <sub>2</sub> uptake, mg. of dry weight		Increase
	Control	With thiamine	
	<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>
1st	9.9	8.6	None
2nd	7.9	8.0	2.0
3rd	6.7	10.8	31.3
4th	4.5	12.0	166.6
5th	2.4	12.0	400
Tissue	Pyruvate utilization in 5 hrs.		
Diaphragm	17.2	26.2	52.2
Rectus	10.0	18.5	85

## DISCUSSION

The metabolism of pyruvate has attracted considerable attention because it is the connecting link between the decomposition and the synthesis of foodstuffs that provide the energy for the chemical activities of the cell. The extreme reactivity of pyruvate (28, 29) makes possible such a rôle. These manifold reactions in which pyruvate takes part, some of them reversible, some ir-

<sup>1</sup> Proper precautions were taken to avoid the effects of bacterial contamination.

reversible, are catalyzed by a variety of enzyme systems. If we leave aside the protein moiety of these enzyme systems, the reversible oxidation-reduction of pyruvate  $\rightleftharpoons$  lactate is catalyzed by diphosphopyridine nucleotide, the reversible amination of pyruvate  $\rightleftharpoons$  alanine is catalyzed by flavin dinucleotide, and the reversible transamination of pyruvate  $\rightleftharpoons$  *l*(+)-glutamate is catalyzed by an as yet unknown enzyme system which is neither pyridine nucleotide nor diphosphothiamine. Diphosphothiamine accelerates the following reactions when pyruvate is one of the reactants, decarboxylation (30), oxidation (31-33), dismutation (31, 33, 34). Quite recently, Silverman and Werkman (35) have shown that it takes part in the condensation of pyruvate and its subsequent decarboxylation and formation of acetylmethylcarbinol. All these reactions have the common property of being irreversible and of being accompanied by decarboxylation. In the experiments reported here, diphosphothiamine has been found to accelerate the synthesis of carbohydrate, citrate,  $\alpha$ -ketoglutarate, acetoacetate, and succinate (Table IX). All these reactions start as condensation products of pyruvate; in all of them there is a step in which an oxidative decarboxylation exists. In the synthesis of carbohydrate, diphosphothiamine-protein may catalyze either the carboxylation of pyruvate-phosphate to phosphoenoloxalacetate or the decarboxylation of this compound to phosphoenolpyruvate (Reaction 2). In the synthesis of citrate and  $\alpha$ -ketoglutarate, diphosphothiamine-protein may act as a condensation enzyme for the condensation of pyruvate with oxalacetate (Reaction 3), since the oxidative decarboxylation of oxalomesaconic acid and of  $\alpha$ -keto- $\beta$ -carboxyglutaric acid seems to proceed spontaneously. In the synthesis of succinate it may act as a condensation enzyme or a carboxylation enzyme, carboxylating pyruvate to oxalacetate which would be reduced to succinate through malate and fumarate (38). In the synthesis of acetoacetate it may act either as an enzyme for the condensation of pyruvate and acetate to acetopyruvate (Reaction 5) or as an oxidation enzyme for the oxidation of the latter to acetoacetate and  $\text{CO}_2$ . Whether diphosphothiamine is the component of a new enzyme system, a condensation enzyme as postulated here, or a component which acts, after the condensation product has been formed, in the oxidative decarboxylation step found in all these

TABLE IX  
*Prosthetic Groups of Activating Proteins of Enzyme Systems for Pyruvate Metabolism*

Prosthetic group of activating protein	Reversibility	Nature of reaction	End-product determined	Reference No.
1. Diphosphopyridine nucleotide.....	Reversible	Oxidation-reduction	Lactate	(36)
2. Flavin dinucleotide.....	"	Oxidation-amination	Alanine	(37)
3. Unknown (not (1) or (4)).....	"	Transamination	"	This paper
4. Diphosphothiamine.....	Irreversible	Decarboxylation	Acetaldehyde + CO <sub>2</sub>	(30)
"	"	Oxidation	Acetate + CO <sub>2</sub>	(31-33)
"	"	Dismutation	Lactate + acetate + CO <sub>2</sub>	(31, 33, 34)
"	"	"	Acetate + formate	(33)
"	"	Condensation	Acetylmethylcarbinol + CO <sub>2</sub>	(35)
"	"	"	Carbohydrate	This paper
"	"	"	Citrate	"
"	"	"	Acetoacetate	"
"	"	"	Succinate	"
"	"	"	α-Ketoglutarate	"

reactions cannot be answered from these experiments. An argument against the postulated hypothesis is that it complicates the function of diphosphothiamine. In fact, Lipmann (13) has suggested that condensation reactions occur rather with the reaction products of pyruvate oxidation, namely acetylphosphate, and that thiamine acts therefore only by increasing the formation of the reactive acetylphosphate. Unfortunately, all attempts to simplify the rôle of diphosphothiamine have met with failure, for decarboxylation, oxidation, and dismutation of pyruvate, all of which are catalyzed by diphosphothiamine, cannot be explained as originating from a single reaction. The second alternative suggestion, that it is a catalyst for the oxidation of the condensation products, would seem untenable if oxidative decarboxylations of long chain keto compounds occur spontaneously, as maintained by many investigators.

The experiments on oxidation and reduction of thiamine and diphosphothiamine show that the chemical passivity of thiamine towards oxidation-reduction is considerably strengthened by phosphorylation, while its activity as an enzyme component is manifested only after phosphorylation. Therefore, diphosphothiamine does not act like the other prosthetic groups (pyridine nucleotides, flavins) which combine with the activating proteins as an oxidation-reduction system. Probably, on firmly combining with the specific proteins, it becomes an integral part of the activating protein of the enzyme systems.

#### SUMMARY

In *in vitro* experiments with tissues from avitaminotic animals it has been found that the addition of thiamine accelerates condensation reactions of pyruvate leading to the synthesis of carbohydrate, of  $\alpha$ -ketoglutarate, of citrate, of acetoacetate, and of succinate. The oxygen consumption and the utilization of pyruvate by the diaphragm and the skeletal muscle of avitaminotic rats were increased on addition of thiamine. The rôle of thiamine in these synthetic processes is discussed.

We wish to express our thanks to Merck and Company, Inc., for a generous grant which allowed the conclusion of these experiments.

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## STUDIES ON BIOLOGICAL OXIDATIONS

### XVII. THE EFFECT OF THIAMINE ON THE METABOLISM of $\alpha$ -KETOGLUTARATE

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$\alpha$ -Ketoglutarate like other  $\alpha$ -keto acids is extremely reactive. In the presence of oxygen it undergoes an oxidative decarboxylation (1, 2); in the absence of oxygen it undergoes dismutation (3), reductive amination (4), or transamination (5, 6). Its utilization by animal tissues and bacteria has been demonstrated, although its rôle in the chemical activities of the organized cells is not yet understood. The mechanism of its decomposition and the nature of the components of the enzyme systems catalyzing such decomposition are not yet known.

From a study of the comparative rates of oxidation of pyruvate and  $\alpha$ -ketoglutarate by different species of bacteria, Barron and Friedemann (7) concluded that the enzyme systems for the oxidation of these  $\alpha$ -keto acids must be different, because some bacteria which oxidized pyruvate with great speed did not utilize  $\alpha$ -ketoglutarate. Since diphosphothiamine is a component of the enzyme systems for the oxidation, dismutation, and condensation of pyruvate, it was plausible to postulate that it might also be a component of the enzyme systems which act in the oxidation and dismutation of  $\alpha$ -ketoglutarate. The stimulating effect of thiamine upon the oxidation and the utilization of  $\alpha$ -ketoglutarate by the tissues of avitaminotic rats supports the opinion that the enzyme system for the oxidative breakdown of  $\alpha$ -ketoglutarate contains diphosphothiamine as one of its components. It is likely that the dismutation of  $\alpha$ -ketoglutarate is also catalyzed by diphosphothiamine, because of its similarity to pyruvate dismuta-

tion which requires diphosphothiamine. Neither the reductive amination nor the transamination is catalyzed by the phosphorylated thiamine.

#### EXPERIMENTAL

The methods and technical details in these experiments were similar to those reported on the rôle of thiamine in pyruvate metabolism (8). Preliminary experiments showed that if thiamine and  $\alpha$ -ketoglutarate were added to the tissue at the same time, the thiamine effect on  $\alpha$ -ketoglutarate oxidation failed to be observed. The experiments became reproducible and the thiamine effect uniform only when thiamine was incubated with the tissue for 30 minutes previous to  $\alpha$ -ketoglutarate addition, the incubation time necessary to allow phosphorylation of thiamine to diphosphothiamine. The use of diphosphothiamine in sliced tissue experiments is not advisable, because it does not penetrate the cell membranes, and because diphosphothiamine dissolved in the buffer systems is rapidly dephosphorylated by the tissue phosphatases. To make certain that the tissues were deficient in thiamine, only rats in the last stages of avitaminosis were used.

Since the first step in the oxidation of  $\alpha$ -ketoglutarate is the formation of succinate ( $\alpha$ -ketoglutarate +  $\frac{1}{2}\text{O}_2 \rightarrow$  succinate +  $\text{CO}_2$ ), the effect of thiamine on the rate of this oxidation by the avitaminotic tissues was studied by inhibiting the further oxidation of succinate with large concentrations of malonate. The oxygen consumption of tissue slices from avitaminotic rats was measured in the presence of 0.03 M malonate, the  $\alpha$ -ketoglutarate being added 30 minutes later. Data for one of these experiments are given in Fig. 1, where the oxygen uptake plotted is that measured 30 minutes after addition of malonate and thiamine. Addition of thiamine to kidney tissue treated with malonate in the absence of substrate produced some increase (28 per cent) in the oxygen consumption, presumably by accelerating the oxidation of the small amounts of pyruvate present or being formed during the metabolism of the tissue carbohydrates. In the presence of  $\alpha$ -ketoglutarate, thiamine increased the oxygen uptake by 48 per cent. The oxidation of  $\alpha$ -ketoglutarate by the kidney, liver, and heart of avitaminotic rats was also increased on addition of thiamine. In chopped brain the effect was very small, 6 per cent increase

(Table I). (The failure of thiamine to raise significantly the oxygen consumption of pigeon brain in the presence of  $\alpha$ -ketoglutarate was previously reported by McGovan and Peters (9).)

Table II shows that the effect of thiamine is directly due to an increased utilization of  $\alpha$ -ketoglutarate. On addition of thiamine,

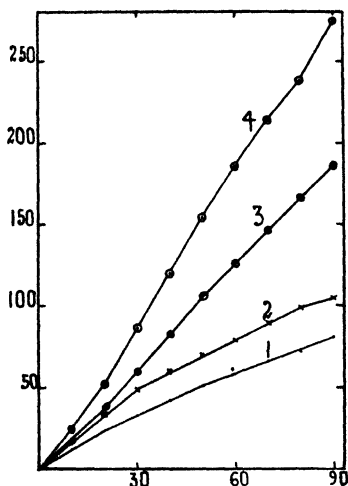


FIG. 1

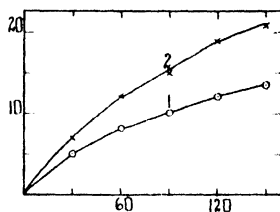


FIG. 2

FIG. 1. Effect of thiamine on the oxidation of  $\alpha$ -ketoglutarate by the kidney of avitaminotic rats. Abscissa, time in minutes; ordinate,  $O_2$  uptake in c.mm. per 100 mg. of tissue; temperature  $38^\circ$ ; pH 7.4. Buffer, Ringer-phosphate. Concentration of malonate, 0.03 M; of  $\alpha$ -ketoglutarate, 0.01 M. Thiamine, 50  $\gamma$ . Curve 1, malonate; Curve 2, malonate + thiamine; Curve 3, malonate + ketoglutarate; Curve 4, malonate + ketoglutarate + thiamine.

FIG. 2. Effect of thiamine on the oxidation of citrate by the heart of thiamine-deficient rats, average of four experiments. Buffer, Ringer-phosphate; temperature  $38^\circ$ . Concentration of citrate, 0.0046 M. Ordinate,  $O_2$  uptake, in c.mm. per mg. of dry weight; abscissa, time in minutes. Curve 1, control; Curve 2, effect of thiamine (50  $\gamma$ ).

the amount of  $\alpha$ -ketoglutarate used by the kidney of avitaminotic rats increased from 3.27 micromoles per mg. per hour to 4.75 micromoles, an increase of 45 per cent. In chopped brain the increase was insignificant, in agreement with the observations on the oxygen consumption.

Citrate in the tissue is changed by aconitase into *cis*-aconitic

and isocitric acids, the isocitric acid being oxidized to the corresponding keto acid ( $\alpha$ -keto- $\beta$ -carboxyglutaric acid), which is

TABLE I

*Effect of Thiamine on Oxidation of  $\alpha$ -Ketoglutarate by Tissues of Thiamine-Deficient Rats*

Concentration of  $\alpha$ -ketoglutarate, 0.01 M; concentration of malonate, 0.03 M; buffer, Ringer-phosphate; pH 7.4.

Tissue	O <sub>2</sub> uptake, per mg. per hr., dry weight		Increase due to thiamine
	Control	With thiamine	
	<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>
Kidney.....	6.8	7.8	14.7
".....	8.1	9.5	17.3
".....	7.2	11.0	52.7
".....	7.4	11.1	50.0
".....	8.4	11.3	34.5
".....	9.8	11.0	10.4
".....	8.4	10.7	27.4
".....	6.3	8.1	28.5
Liver.....	4.4	5.6	27.2
".....	4.9	5.5	12.2
Heart, ventricle.....	7.3	8.4	15.0
" ".....	6.3	12.8	103.0
Brain, chopped, per 100 mg.....	113.5	120.6	6.3

TABLE II

*Effect of Thiamine on Utilization of  $\alpha$ -Ketoglutarate by Thiamine-Deficient Tissues (Rat)*

Buffer, Ringer-phosphate; pH 7.4; incubation time, 120 minutes; concentration of  $\alpha$ -ketoglutarate, 0.01 M; thiamine, 50  $\gamma$ . The figures represent micromoles per mg. of dry tissue.

Tissue	$\alpha$ -Ketoglutarate utilization	
	Control	With thiamine
Kidney.....	3.60	5.24
".....	2.14	3.01
".....	4.07	6.00
Brain.....	1.96	1.97

unstable and splits off CO<sub>2</sub> spontaneously to give  $\alpha$ -ketoglutaric acid (10). The oxidation system contains, according to von Euler

*et al.* (11), an activating protein and triphosphopyridine nucleotide. If diphosphothiamine is the prosthetic group of the activating protein of  $\alpha$ -ketoglutarate oxidase, there should be an increase in the oxygen uptake of avitaminotic tissues in the presence of citrate upon the addition of thiamine, because of increased oxidation of the  $\alpha$ -ketoglutarate formed from citrate. Experiments with the heart (slices) of avitaminotic rats confirmed the validity of this assumption. In the presence of citrate, addition of thiamine actually increased the oxygen consumption by 50 per cent (Fig. 2).

Since all these effects were obtained only after an incubation period of thiamine with the tissues, it is plausible to postulate that diphosphothiamine is one of the components of  $\alpha$ -ketoglutarate oxidase, for Ochoa (12) has demonstrated that thiamine is phosphorylated by tissues from avitaminotic animals. It has already been shown (8) that diphosphothiamine does not act in transaminations. The rôle of thiamine in the dismutation of  $\alpha$ -ketoglutarate is now being studied.

#### SUMMARY

The oxidation and utilization of  $\alpha$ -ketoglutarate by the tissue of avitaminotic rats were increased on addition of thiamine. Since this effect was observed only when thiamine was previously incubated with the tissue to make its phosphorylation possible, it is concluded that the activating protein of  $\alpha$ -ketoglutarate oxidase is, like that of pyruvate oxidase, a diphosphothiamine-protein.

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## STUDIES ON BIOLOGICAL OXIDATIONS

### XVIII. THE CITRIC ACID CYCLE IN PIGEON MUSCLE RESPIRATION

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(Received for publication, June 30, 1941)

Since its proposal in 1937 the significance of the citric acid cycle in carbohydrate metabolism has been actively discussed by many workers. The protagonists for the cycle claim that it is the main pathway for carbohydrate metabolism in pigeon breast muscle (1), that it plays a significant rôle in the metabolism of pigeon liver (2), and that it functions in the metabolism of mammalian tissues (3). However, some investigators have questioned the importance, and even the existence, of a cycle containing citric acid in pigeon breast muscle (4-6). Such a cycle does not appear to function in the respiration of pigeon brain (7). One of us recently reviewed the evidence for and against the citric acid cycle (8). Quite recently, Wood *et al.* (9) employing heavy carbon as a tracer have also questioned the formation of citrate in the synthesis of  $\alpha$ -ketoglutarate from pyruvate by liver.

In the course of other work performed in this laboratory upon animal tissues, results were obtained which appeared difficult to reconcile with the citric acid cycle. Because the data secured might have been due in part to differences between mammalian tissues and pigeon breast muscle, we have employed the latter tissue in a study of the relative rates of utilization of citrate and pyruvate under various conditions. These have been compared directly with the rates of oxygen consumption determined as in the original experiments of Baumann and Stare (6) which indicated that citrate differed from the other members of the cycle in being a much weaker catalyst, particularly in the presence of



malonate. The latter observation has been confirmed by Smyth (10).

Our data show that the conditions required for the existence of the citrate cycle are not fulfilled in chopped avian muscle. Accordingly, it appears that citric acid is of only minor importance in the carbohydrate metabolism of pigeon breast muscle.

#### EXPERIMENTAL

All experiments were performed with minced pigeon breast muscle. After decapitation, the breast muscle was rapidly removed, briefly rinsed with ice water, cooled for a minute between ice cubes, and minced in a cold Latapie mincer. The mince was collected in a cold Petri dish lined with several layers of filter paper moistened with cold physiological saline. The tissue samples, approximately 200 mg. of wet weight, were weighed separately on cellophane on a torsion balance, the tissue and the cellophane placed in the manometric flasks, and the tissue dispersed by means of a wire. A Ringer-phosphate buffer free of calcium, pH 7.4, was used (11). It was made up in 2 N concentration and diluted either with water or supplementary solutions. The contents of each vessel totaled 2.8 ml. All supplementary solutions were made up fresh for each experiment and were neutralized to pH 7.4. The oxygen uptake was measured at 38° in the usual way in a Warburg apparatus. At the end of the experiment the contents of the manometric vessels were analyzed for citrate and pyruvate, although in some series separate samples for analysis were set up in Erlenmeyer flasks, with twice the volume and twice the amount of tissue as with the manometric vessels. The details of the pyruvate and citrate determinations are the same as in Paper XVI (12). All results are reported in terms of micromoles per 100 mg. of tissue, dry weight.

#### *Results*

Table I shows the effects of various additions upon the utilization of pyruvate. In unsupplemented muscle, pyruvate utilization was quite high and was increased by the addition of those compounds which stimulate oxygen consumption; that is, citrate,  $\alpha$ -ketoglutarate, succinate, and fumarate. Malonate in this experiment inhibited pyruvate utilization by 56 per cent, the inhibition being completely overcome by addition of equal concen-

trations of succinate, fumarate, or  $\alpha$ -ketoglutarate. Citrate was less effective than the other compounds. Particular emphasis should be placed on the fact that it was less effective than  $\alpha$ -ketoglutarate. The addition of fumarate to the vessel containing citrate plus malonate completely restored the pyruvate utilization.

Table II shows that citrate utilization does not parallel pyruvate utilization, the rate of pyruvate utilization being more than twice as great as that of citrate. Under these circumstances considerable citrate should have accumulated. Yet of the 16.0 micromoles of pyruvate which disappeared, only 1.6 could be accounted for as synthesized citrate. Since the tissue was capable of utilizing 6.2

TABLE I  
*Utilization of Pyruvate by Respiring Pigeon Breast Muscle in  
Presence of Various Supplements*

The values are expressed as micromoles of pyruvate utilized per 100 mg. of tissue (dry weight) in 2 hours. All supplements are present in a final concentration of 0.005 M, except pyruvate which is 0.007 M.

Supplements (in addition to pyruvate which is present in all vessels)	Pyruvate change
None.....	-16.3
Succinate.....	-18.5
Fumarate.....	-21.4
Citrate.....	-20.2
$\alpha$ -Ketoglutarate.....	-16.8
Malonate.....	-7.2
Succinate + malonate.....	-22.0
Fumarate + ".....	-23.3
Citrate + ".....	-12.4
$\alpha$ -Ketoglutarate + malonate.....	-17.5

micromoles of citrate, and under these conditions actually only 1.6 micromoles were formed, there remained 8.2 micromoles of pyruvate, or approximately 50 per cent of the added pyruvate, which disappeared and could not be accounted for by the citric acid cycle.

The rate of citrate utilization was independent of the presence of pyruvate. This is another fact that cannot be explained by the citrate cycle, for since citrate was utilized more slowly than pyruvate, the addition of pyruvate should have resulted in a diminished utilization of the added citrate. Citrate utilization was increased by the addition of malonate both with and without added pyruvate, and yet malonate inhibited pyruvate utilization.

The mechanism of this enhancing effect of malonate on citrate utilization is unknown. The removal of citrate was diminished in the presence of fumarate plus malonate, and in the presence of malonate, fumarate, and pyruvate; the removal of pyruvate was increased in the presence of fumarate. These data cannot be explained on the basis that citrate synthesis is increased when fumarate and pyruvate are added to tissue in the presence of malonate, since control experiments showed only 2 micromoles of citrate to be synthesized under these conditions, whereas fumarate decreased citrate utilization by 6 micromoles.

TABLE II

*Utilization of Pyruvate and Citrate by Respiring Pigeon Breast Muscle in Presence of Various Supplements*

The values are expressed as micromoles utilized per 100 mg. of tissue (dry weight) in 2 hours. The supplements are present in a final concentration of 0.005 M, except pyruvate which is 0.007 M.

Supplements	Pyruvate change	Citrate change
Pyruvate.....	-16.0	+1.6
Citrate + pyruvate.....	-15.8	-7.9
“ no pyruvate added.....		-6.2
Malonate + pyruvate.....	-8.8	
Citrate + malonate + pyruvate.....	-12.0	-9.4
“ + “ no pyruvate added.....		-9.8
“ + “ + fumarate + pyruvate...	-18.6	-3.6
“ + “ + “ no pyruvate added.....		-3.5

In Table III are the results of a representative experiment in which pyruvate and citrate utilization were determined on the contents of manometric vessels at the conclusion of the measurement of oxygen consumption. With 0.007 M pyruvate, malonate alone inhibited respiration markedly, citrate was without effect, and the other supplements increased it. In addition, fumarate, succinate, and  $\alpha$ -ketoglutarate produced marked increases in respiration even in the presence of malonate, whereas respiration was appreciably less than normal in the presence of citrate plus malonate.

Similar differences between supplements were observed when the rate of utilization of pyruvate was measured. Succinate,

fumarate, and  $\alpha$ -ketoglutarate, in the absence of malonate, increased pyruvate utilization, but citrate had no effect. In the presence of malonate, which inhibited pyruvate utilization to the extent of 88 per cent, succinate, fumarate, and  $\alpha$ -ketoglutarate increased the utilization above the normal control, whereas citrate was decidedly inferior. Since  $\alpha$ -ketoglutarate markedly increased the rate of pyruvate utilization in the presence of malonate, and citrate was less efficient, it appears that the pathways by which pyruvate is utilized lead more directly to  $\alpha$ -ketoglutarate than to citrate. This possibility has already been suggested by Krebs and Eggleston (13), but dismissed as improbable.

TABLE III

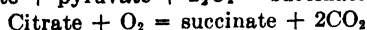
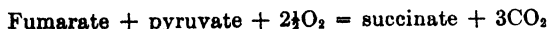
*Respiration of Pigeon Breast Muscle and Utilization of Pyruvate and Citrate in Presence of Various Supplements*

The oxygen uptake and utilization are expressed in micromoles per 100 mg. of tissue (dry weight) in 1 hour. All supplements are present in a final concentration of 0.005 M, except pyruvate which is 0.007 M.

Supplements (in addition to pyruvate which is present in all vessels)	Oxygen uptake	Pyruvate change	Citrate change
None.....	26.3	-8.4	
Malonate.....	7.15	-1.0	+1.0
Citrate.....	21.0	-7.9	-5.6
“ + malonate.....	15.6	-5.8	-7.1
“ + “ + fumarate...	43.8	-14.0	-2.2
$\alpha$ -Ketoglutarate.....	42.9	-10.8	
“ + malonate.....	40.6	-9.7	
Succinate.....	47.7	-16.7	
“ + malonate.....	39.3	-13.1	
Fumarate.....	46.5	-13.2	+1.6
“ + malonate.....	50.9	-16.0	+1.4

## DISCUSSION

The proponents of the citrate cycle point out that in the presence of malonate, fumarate would be expected to have a greater stimulating effect upon pyruvate utilization and oxygen uptake than citrate. Thus,



If this argument were valid, then  $\alpha$ -ketoglutarate should be even less effective than citrate ( $\alpha$ -ketoglutarate +  $\frac{1}{2}\text{O}_2 = \text{succinate} +$

CO<sub>2</sub>), while succinate should have no effect at all. Actually, we find that all the members of the citrate cycle *except citrate* are approximately equally effective in restoring the oxygen uptake and pyruvate utilization of muscle in the presence of malonate. Since malonate does not inhibit citrate utilization but rather enhances it, the failure to obtain stimulation of oxygen uptake and increased pyruvate utilization cannot be attributed to inhibition of the isocitric oxidase system with malonate. We are left with the conclusion that the rôle of citrate in pyruvate metabolism and in respiration is a lesser one than is that of the other members of the cycle.

Pyruvate is utilized by pigeon breast muscle more than 3 times as rapidly as citrate. If the main pathway of pyruvate utilization were through citrate, one would expect citrate to accumulate in quantities which approximate the difference in the rates of utilization of the two compounds. Yet only insignificant amounts of citrate accumulated when pyruvate was utilized. We are therefore again led to the conclusion that pyruvate is not converted to citrate in the course of its oxidation by pigeon breast muscle mince respiring in 0.007 M pyruvate.

In the presence of malonate, citrate utilization did not parallel oxygen uptake but rather was inverse to it. This could not be explained on the basis of increased citrate synthesis in rapidly respiring tissues, since citrate synthesis was found to be far less than the difference in utilization.

Our experimental observations appear to favor a cycle involving a conversion of pyruvic acid to  $\alpha$ -ketoglutarate, without citrate as an intermediary, followed by the Szent-Györgyi series of conversions of the dicarboxylic acids to oxalacetate. The occasional catalysis of respiration observed when citrate is added to muscle is probably not due to citrate itself but rather to the C<sub>4</sub> acids and  $\alpha$ -ketoglutarate which may be formed from it. Citrate may serve as a "stock-room" for the essential catalysts, and exert an effect on respiration only when they are low.

#### SUMMARY

1. The uptake of oxygen and utilization of pyruvate were inhibited approximately 70 per cent by 0.005 M malonate. Oxygen uptake and pyruvate utilization were restored in the presence of such a malonate concentration when equimolar concentrations of

$\alpha$ -ketoglutarate, succinate, or fumarate were added. However, citrate did not restore either oxygen consumption or pyruvate utilization in the presence of malonate. It thus appeared that the oxygen uptake and pyruvate utilization in the presence of malonate were not connected with a citrate reaction.

2. The rate of pyruvate utilization was greater than that of citrate utilization, did not parallel the latter, and frequently was not even affected by added citrate.

3. The addition of malonate inhibited respiration and pyruvate utilization, but accelerated citrate utilization. Fumarate added to muscle in the presence of malonate increased oxygen uptake and pyruvate utilization, but decreased citrate utilization. This observation could not be accounted for by an increased synthesis of citrate in rapidly respiring tissue.

4. It is therefore doubtful whether citrate represents an essential stage in the utilization of pyruvate and in the respiration of pigeon breast muscle.

We wish to express our sincere appreciation to Dr. E. S. Guzman Barron for the facilities of his laboratory and for his constructive criticism and suggestions; and to Dr. Carl Baumann, Department of Biochemistry, University of Wisconsin, for helpful suggestions and for criticism of this manuscript.

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## LETTERS TO THE EDITORS

### THE EFFECT OF PURINES ON THE ACTION OF SULFONAMIDES

Sirs:

When *Escherichia coli* is grown in a basal medium of inorganic salts and glucose, the addition of methionine antagonizes the action of the sulfonamides.<sup>1,2</sup> Using methods described elsewhere,<sup>2</sup> we find this methionine effect to be increased by the addition of either xanthine or guanine (see Line C' of the table for typical results). On the other hand, in the absence of methionine these purines increase the action of sulfanilamide (Line B). In the basal medium, with (A') or without (A) methionine, these purines have no effect on growth. Such contrasting effects in the sulfanilamide-treated organism suggest metabolic relationships, as yet unknown, for sulfanilamide, methionine, and the purines.

Time in hours for *Escherichia coli* to reach a density of 132 million per ml. from an inoculum of 20 per ml. in a medium of inorganic salts and glucose at 37°. In the uninhibited culture, growth is exponential for practically the entire period.

	Added to basal medium		Effect of added purine		
	Sulfanilamide	dl-Methionine	No purine	Hypoxanthine, 10 <sup>-4</sup> M	Xanthine, 10 <sup>-4</sup> M
	M	M	hrs.	hrs.	hrs.
A	0	0	19	18	20
A'	0	10 <sup>-4</sup>	16.5	16	16
B	10 <sup>-4</sup>	0	38	>132	106
C	10 <sup>-3</sup>	0	>110	>110	>110
C'	10 <sup>-3</sup>	10 <sup>-4</sup>	83	>110	34
D (resistant strain)	0	0	21	38	21
D' " "	0	10 <sup>-4</sup>	17	17	16

<sup>1</sup> Kohn, H. I., and Harris, J. S., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **133**, 354 (1941). Bliss, E. A., and Long, P. H., *Bull. Johns Hopkins Hosp.*, **69**, 14 (1941).

<sup>2</sup> Harris, J. S., and Kohn, H. I., *J. Pharmacol. and Exp. Therap.*, **73**, in press.



Hypoxanthine and adenine increase the inhibition caused by sulfanilamide in the basal medium with (C') or without (B) methionine. However, in the absence of sulfanilamide (A, A'), these 6-purines are without effect upon the rate of growth.

The ability to increase the antisulfanilamide action of methionine evidently depends upon substitution at the 2 position of the purine, whereas the intensification of sulfanilamide action is determined at the 6 position. When *p*-aminobenzoate completely nullifies the action of sulfanilamide, all these purines are without effect.

The growth of organisms made resistant to  $10^{-2}$  M sulfanilamide (by continued subculture in increasing amounts of the drug) is inhibited by hypoxanthine or adenine, but not by xanthine or guanine (D). Thus the development of resistance is accompanied by a change in the response of the organisms to the purines. The addition of sulfanilamide does not alter this effect, whereas  $10^{-7}$  M *p*-aminobenzoate or  $10^{-4}$  M methionine (D') completely abolishes it.

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HENRY I. KOHN

Received for publication, October 24, 1941

## NON-CRYSTALLIZABLE VITAMIN A\*

Sirs:

Repeated bioassays of pure crystalline vitamin A (m.p. 63–64°)<sup>1</sup> against U.S.P. reference oil have yielded potency values ranging around 4,500,000 U.S.P. units per gm.<sup>2</sup> Since the average extinction coefficient ( $E_{1\text{ cm.}}^{1\%}$ , 328 m $\mu$ ) of the crystalline vitamin is 1725, the conversion factor is 2600. This average factor of 2600 was considerably higher than the value 2000, accepted for most fish oils, so that it became of interest to determine the factor or relative potency of the vitamin A left in the mother liquor. The vitamin A in the filtrate gave an ultraviolet spectrum apparently identical with that of the crystals, but it proved refractory to crystallization.

The liver oil of the ling cod (*Ophiodon elongatus*) was distilled and the distillate containing approximately 29 per cent vitamin A esters ( $E_{1\text{ cm.}}^{1\%}$ , 328 m $\mu$  = 500) was saponified. The free vitamin A in the resulting concentrate was crystallized from ethyl formate by the method previously described.<sup>1</sup> The crystallization mother liquor was then evaporated under reduced pressure and the residue redistilled to carry the concentration of the vitamin A as far as possible. Only a small amount of the vitamin A in this distillate could be crystallized from ethyl formate; therefore the mother liquor was evaporated and the crystallization residue examined. It contained about 46 per cent of the initial vitamin A and was an orange-red oil with an extinction coefficient ( $E_{1\text{ cm.}}^{1\%}$ , 328 m $\mu$ ) of 1210. The spectrophagic curve was very slightly broader than that of crystalline vitamin A and the absorption maximum occurred at 330 m $\mu$ .

Aliquots of the uncrystallizable residue were diluted and assayed with the results given in the tabulation.

\* Communication No. 29 from the Laboratories of Distillation Products, Inc.

<sup>1</sup> Baxter, J. G., and Robeson, C. D., *Science*, **92**, 203 (1940).

<sup>2</sup> Unpublished data.

	Amount fed daily	Average growth response	Equiva- lency, u.s.p. units per day	Potency, u.s.p. units per gm.	Conversion factor
	<i>mg.</i>	<i>gm.</i>			
Crystallization residue ( $E_{1\text{ cm.}}^{1\%} = 9.22$ )	0.0701	<12	<1.00	<14,300	<1550
“ “	0.1402	36.3	1.92	13,700	1490

The bioassay procedure employed was that detailed in the U.S.P. XI except that greater precision was obtained by running three different levels of U.S.P. reference oil with each assay, determining the dose-response relationship for this standard material, and calculating from it the "equivalency" of all levels of test materials in terms of U.S.P. units fed daily.

It is evident from the above that a fish liver oil has yielded two preparations of vitamin A having similar ultraviolet absorption spectra, but conversion factors (relative biological potencies) of 2600 and 1500. These factors differ from the generally accepted value of 2000 for the whole oil. The existence of more than one variety of vitamin A, spectrally identical but different in crystallizability and potency, if generally substantiated, will help to explain many paradoxes in past vitamin A research. Natural oils which have been reported as having significantly different conversion factors may have possessed different proportions of these vitamin A's or inactive material absorbing sharply at 328  $m\mu$ . The preparation of concentrates, by distillation or extraction, which showed conversion factors different from those of the original oils from which they were prepared, may have concentrated the two vitamin A's differently or destroyed one form preferentially.

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# THE SIGNIFICANCE OF THE RATIO OF LACTIC TO PYRUVIC ACID IN THE BLOOD AFTER EXERCISE

Sirs:

Previous workers<sup>1</sup> have shown that the concentration of pyruvic acid increases greatly after muscular exercise and then diminishes as the lactic acid content of the blood returns to normal. In our

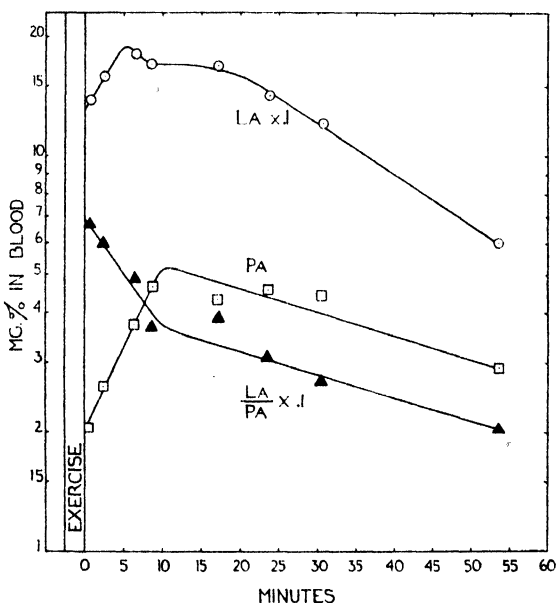


FIG. 1. The subject ran down and then up seventeen flights of steps in 2 minutes and 40 seconds. Maximum in blood from arm veins, lactic acid 188 mg. per cent, pyruvic acid 5.20 mg. per cent.

experiments the concentration of pyruvic acid rises and declines logarithmically, or nearly so, with time. Following severe muscular exertion (Fig. 1), the ratio of lactic to pyruvic acid, which

<sup>1</sup> Johnson, R. E., and Edwards, H. T., *J. Biol. Chem.*, **118**, 427 (1937).  
Lu, G. D., and Platt, B. S., *Biochem. J.*, **33**, 1538 (1939).

at first is extremely high, 69 in the experiment cited, falls rapidly during the 1st few minutes after exercise, and then decreases slowly. The point of inflection of the curve occurs at from 4 to 10 minutes, depending upon the severity of the exertion. At this time the CO<sub>2</sub> capacity and pH approach their minimum<sup>2,3</sup> and lactic<sup>2</sup> and pyruvic acids attain their maximum concentration in the blood. Of particular interest is the fact that at about this time the pulse, the respiration, the oxygen intake,<sup>4</sup> and the oxygen content of the blood are rapidly returning to normal. According to Margaria, Edwards, and Dill,<sup>5</sup> the "alactacid oxygen debt" is restored within the first 5 minutes or so.

*Analysis after Light Exercise of Whole Blood from Arm Veins*

Time after exercise	Lactic acid	Pyruvic acid	Lactic Pyruvic
min. sec.	mg. per cent	mg. per cent	
1 8	57.0	2.64	21.5
8 40	46.4	3.11	15.0
30 0	30.0	1.93	15.5
60 0	19.6	1.28	15.3

*These and other considerations lead us to believe that the ratio is an indicator of returning normal oxidative conditions. That this is probably so is suggested by results obtained after mild exercise (see the table). In such experiments, despite a marked rise of the lactic and pyruvic acids, the ratio rapidly returns to the normal ratio of 12 to 15.*

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<sup>2</sup> Barr, D. P., Himwich, H. E., and Green, R. P., *J. Biol. Chem.*, **55**, 495 (1923).

<sup>3</sup> Barr, D. P., and Himwich, H. E., *J. Biol. Chem.*, **55**, 525, 539 (1923).

<sup>4</sup> Laug, E. P., *Am. J. Physiol.*, **107**, 687 (1934).

<sup>5</sup> Margaria, R., Edwards, H. T., and Dill, D. B., *Am. J. Physiol.*, **106**, 689 (1933).

## CATALYZED HYDROLYSIS OF AMIDE AND PEPTIDE BONDS IN PROTEINS

*Sirs:*

It has been shown that titration curves of at least two proteins, wool and egg albumin, when obtained with different strong acids, are not identical but occupy distinct positions with respect to the pH coordinate, depending on the acid.<sup>1</sup> These wide differences were interpreted in terms of combination of the protein with anions as well as with hydrogen ions, the combination with each anion being governed by its own distinct dissociation constant. These constants, calculated from the titration data, differ over a several thousandfold range. It has now been found that the velocities of hydrolysis at 65° of these proteins by dilute solutions of these acids (less than 0.1 M) also differ very widely. These differences have been chiefly studied by measuring the rate of liberation of ammonia by hydrolysis of amide linkages, since this directly measures the rate at which single bonds are attacked. However, very similar differences are found when the hydrolysis is followed by measuring the rate at which all forms of soluble nitrogen are liberated from both proteins.

The rates observed with different acids vary over more than a hundredfold range. By using the most effective of the anions studied (dodecylsulfate or dodecylsulfonate, tetradecylsulfate, diphenylbenzenesulfonate, the dye Orange II) as their sodium salts added to solutions of any of the least effective acids, the effects of varying anion and hydrogen ion concentration separately may be studied. It has been found with wool that relatively low concentrations of the "effective" anion suffice to produce a maximum rate when the reaction is followed by measuring ammonia production. No such limit is observed as the hydrogen ion concentration is varied. Thus adding very small amounts

<sup>1</sup> Steinhardt, J., *Ann. New York Acad. Sc.*, **41**, 287 (1941). Steinhardt, J., Fugitt, C. H., and Harris, M., *J. Research Nat. Bur. Standards*, **26**, 293 (1941) RP1377.

of these salts to any dilute solution of hydrochloric acid increases the rate of hydrolysis of both amide and peptide bonds about 100 times, an effect very similar to that produced by adding a smaller amount of the enzyme, pepsin.

The amounts of effective anion producing the maximum rate correspond closely with stoichiometric equivalence to the sum of the strongly basic groups plus the primary amide groups. This correspondence suggests a hydrolytic mechanism requiring combination of amide groups with hydrogen ions. Combination of amide groups, despite their low base strength, with hydrogen ions at these relatively low acidities is possible, because the concurrent combination with tightly bound anions permits the acceptance of a proton without a simultaneous increase in the positive charge on the protein. The resulting susceptibility to acid catalysis thus shows a dependence on the affinity of the anion for protein rather than on the strength of the acid.

A different situation is found when the rate of production of all soluble products rather than of ammonia alone is followed. The rate increases with increasing anion and hydrogen ion concentration beyond the limit yielding the maximum rate of ammonia production. This difference, indicating that peptide groups are less strongly basic than amide groups (or have less affinity for anion), is of potential use in analysis. By using low concentrations of "effective" anions at moderate acidities, practically complete hydrolysis of amide groups may be attained with little general hydrolysis of the protein.

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## DESTRUCTION OF THIAMINE BY A SUBSTANCE IN CERTAIN FISH

Sirs:

Green, Carlson, and Evans<sup>1</sup> recently have shown that foxes developed a severe disease when fed a diet containing raw carp and that this disease could be prevented by the feeding of thiamine. Since studies of the egg white injury factor and its reaction with biotin<sup>2</sup> were in progress in this laboratory at the time that the paper of Green *et al.* appeared,<sup>3</sup> the possibility was considered that an "antithiamine" existed whose relation to thiamine was similar to that of the antibiotin factor to biotin. An attempt was therefore made to demonstrate an antithiamine in carp by chemical and biological procedures.

A freshly killed carp was ground and suspended in water, and 4 volumes of ethanol were added. The alcohol extract was found to contain no thiamine when examined by the method of Emmett *et al.*<sup>4</sup> Furthermore, thiamine added to the fish could not be recovered. 100 gm. of various carps caused the disappearance of 150 to 190  $\gamma$  of the vitamin when 200  $\gamma$  were added. A suspension of carp which had been heated to the boiling point and cooled removed only half as much thiamine. Heating for 15 minutes at 15 pounds pressure destroyed all activity. Similar behavior was observed with solutions of the factor obtained as described below. Aqueous extracts were only one-fourth as active as the suspension of carp, while aqueous extracts of dialyzed suspensions were inactive toward thiamine. However, the active ingredient was not dialyzable. It could be extracted from the insoluble portion of the fish with 10 per cent NaCl.

<sup>1</sup> Green, R. G., Carlson, W. E., and Evans, C. A., *J. Nutrition*, **21**, 243 (1941).

<sup>2</sup> Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **140**, 535 (1941).

<sup>3</sup> Woolley, D. W., and Longsworth, L. G., *J. Biol. Chem.*, in press.

<sup>4</sup> Emmett, A. D., Peacock, G., and Brown, R. A., *J. Biol. Chem.*, **135**, 131 (1940).



One-fourth of the activity of a fish was in the head, three-eighths in the viscera, and three-eighths in the torso.

An independent method of analysis was sought in order to establish these observations more securely and to extend them. After the examination of the responses of a number of micro-organisms it was found that the yeast *Endomyces vernalis* was suited to the purpose. When this organism was inoculated into the medium described by Snell *et al.*,<sup>5</sup> from which thiamine was omitted, slight growth occurred. When thiamine was added, growth was as good as in a malt extract medium.<sup>6</sup> By the use of a quantitative method based on these observations it was found that the aqueous extract of carp contained only 3  $\gamma$  of thiamine and its pyrimidine and thiazole halves per 100 gm. of fish and that suspensions or NaCl extracts of carp destroyed thiamine.

The mode of destruction has not been established. Since the pyrimidine and thiazole halves together were as active as thiamine for *Endomyces* and since thiamine activity for this organism was destroyed by the preparation, the mode of action cannot be to split the molecule into these halves. The destruction was not instantaneous. A certain preparation destroyed no thiamine in zero time, 1.3  $\gamma$  in 6 hours, 4.0  $\gamma$  in 16 hours, 4.3  $\gamma$  in 24 hours, at 25° (*Endomyces* method). Whether the action is an enzymic degradation or a slow formation of a thiamine-antithiamine complex has not yet been established.

1 mg. of the most active preparation thus far obtained by methods suggested above destroyed 1.8  $\gamma$  of thiamine in 2 hours at 25°.

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<sup>5</sup> Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).

<sup>6</sup> Woolley, D. W., *J. Biol. Chem.*, **140**, 453 (1941).

## ABSENCE OF METHIONINE IN CRYSTALLINE HORSE SERUM ALBUMIN

*Sirs:*

The distribution of the sulfur amino acids in purified preparations of the serum proteins has as yet not been determined. A preparation of horse serum albumin (HSA) was obtained through the courtesy of Mr. Manfred Mayer of this Department. It had been crystallized according to the method of Adair and Robinson,<sup>1</sup> recrystallized five times, then dialyzed until free from ammonium sulfate, and finally dried *in vacuo* at room temperature. Such preparations are very low in carbohydrate and glucosamine, similar to HSA Fraction B of Kekwick.<sup>2</sup>

Air-dried HSA was used for analysis and the values corrected for moisture content<sup>3</sup> (9.63 per cent). The total S was 1.82 per cent (ash, 0), of which 0.15 per cent was sulfate S, leaving a protein S content of 1.68 per cent (corrected<sup>3</sup> for sulfate, 0.5 per cent). The total N (micro-Dumas) was 15.90 per cent (corrected for sulfate<sup>3</sup>).

Methionine was completely absent. The protein S was quantitatively accounted for by cystine + cysteine S (1.68 per cent (corrected) in HI hydrolysates<sup>3</sup>). In HCl hydrolysates the separate determination of cystine and cysteine yielded 0.50 per cent (corrected) of cysteine and 5.78 per cent (corrected) of cystine, or 6.28 per cent of cystine + cysteine (1.68 per cent S).

From the distribution of cysteine and cystine the minimum molecular weight of HSA can be calculated on the assumption that the material analyzed is a pure chemical individual (along the lines developed for chymotrypsinogen<sup>3</sup>). HSA contains three residues of cysteine and thirty-four half cystine residues; the

<sup>1</sup> Adair, G. S., and Robinson, M. E., *Biochem. J.*, **24**, 994 (1930).

<sup>2</sup> Kekwick, R. A., *Biochem. J.*, **32**, 552 (1938).

<sup>3</sup> For details see the analysis of chymotrypsinogen (Brand, E., and Kassell, B., *J. Gen. Physiol.*, **25**, 167 (1941)).

calculated minimum molecular weight of 70,700 is in close agreement with physicochemical determinations of the molecular weight.<sup>2</sup>

HSA is an unusual protein in that it contains no methionine. The only other known proteins without methionine are insulin and the oxytocic-pressor hormone from the posterior pituitary.<sup>4</sup> Crystalline human serum albumin of Kendall,<sup>5</sup> on the other hand, contains methionine.<sup>6</sup> Dog serum albumin will be investigated in view of the finding of Whipple *et al.*<sup>7</sup> that in dogs methionine is ineffective in serum protein regeneration.

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<sup>4</sup> van Dyke, H. B., Chow, B. F., Greep, R. O., and Rothen, A., *Am. J. Physiol.*, **133**, 473 (1941).

<sup>5</sup> Kendall, F. E., *J. Biol. Chem.*, **138**, 97 (1941).

<sup>6</sup> Brand, E., Kassel, B., and Kendall, F. E., unpublished experiments.

<sup>7</sup> Madden, S. C., Noehren, W. A., Waraich, G. S., and Whipple, G. H., *J. Exp. Med.*, **69**, 721 (1939).

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